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APPLICATION NUMBER: ***60/486,694***
FILING DATE: ***July 11, 2003***
RELATED PCT APPLICATION NUMBER: ***PCT/US04/11988***

Certified by

Jon W Dudas

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EXPRESS MAIL NO. EV284452776US

PTO/SB/16 (05-03)
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request f r filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Jay	**Nelson**	**Tualitin, Oregon**

☒ *Additional inventors are being named on the* 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

INHIBITION OF FLAVIVIRUS REPLICATION BY INHIBITORS OF *SRC*-FAMILY KINASES

16235 U.S. PTO
60/486694
07/11/03

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ENCLOSED APPLICATION PARTS ***(check all that apply)***

☒ Specification *Number of Pages* 30

☒ Drawing(s) *Number of Sheets* 14

☐ Application Data Sheet. See 37 CFR 1.76

☐ CD(s), Number

☒ Other (specify) **Fee Transmittal (+ copy)**

6 sheets **Sequence Listing (printed only), Supplemental Information, Check**

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.

☒ A check or money order for $80 is enclosed to cover the filing fees.

☐ The Commissioner is hereby authorized to charge filing fees to Deposit Account Number: **04-0258**

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____.

Respectfully submitted,			
SIGNATURE	[signature]	DATE	**July 11, 2003**
TYPED or PRINTED NAME	**Barry L. Davison**	REGISTRATION NO. *(if appropriate)*	**47,309**
TELEPHONE	**206-628-7621**	DOCKET NUMBER:	**49321-97**

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PROVISIONAL APPLICATION COVER SHEET

Additional Page

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	49321-97	Type a plus sign (+) inside this box →	+

INVENTOR(S)/APPLICANT(S)

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Alec	Hirsch	Portland, Oregon
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FEE TRANSMITTAL for FY 2003

Effective 01/01/2003. Patent fees are subject to annual revision.

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 80

Complete if Known	
Application Number	To be assigned
Filing Date	July 10, 2003
First Named Inventor	Nelson
Examiner Name	
Art Unit	
Attorney Docket No.	49321-97

07/11/03 12236 U.S. PTO

METHOD OF PAYMENT *(check all that apply)*

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Deposit Account Number: 04-0258

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FEE CALCULATION

1. **BASIC FILING FEE**

Large Entity Fee Code	Fee($)	Small Entity Fee Code	Fee($)	Fee Description	Fee Paid
1001	750	2001	375	Utility filing fee	
1002	330	2002	165	Design filing fee	
1003	520	2003	260	Plant filing fee	
1004	750	2004	375	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	80

SUBTOTAL (1) ($)80

2. **EXTRA CLAIM FEES**

			Extra Claims		Fee from below		Fee Paid
Total Claims		- 20** =		x		=	
Independent Claims		- 3** =		x		=	
Multiple Dependent						=	

Large Entity Fee Code	Fee ($)	Small Entity Fee Code	Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	84	2201	42	Independent claims in excess of 3
1203	280	2203	140	Multiple dependent claim, if not paid
1204	84	2204	42	** *Reissue independent claims over original patent*
1205	18	2205	9	** *Reissue claims in excess of 20 and over original patent*

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FEE CALCULATION (continued)

3. **ADDITIONAL FEES**

Large Entity Fee Code	Fee ($)	Small Entity Fee Code	Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet.	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	410	2252	205	Extension for reply within second month	
1253	930	2253	465	Extension for reply within third month	
1254	1,450	2254	725	Extension for reply within fourth month	
1255	1,970	2255	985	Extension for reply within fifth month	
1401	320	2401	160	Notice of Appeal	
1402	320	2402	160	Filing a brief in support of an appeal	
1403	280	2403	140	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive – unavoidable	
1453	1,300	2453	650	Petition to revive – unintentional	
1501	1,300	2501	650	Utility issue fee (or reissue)	
1502	470	2502	235	Design issue fee	
1503	630	2503	315	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Petitions related to provisional applications	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	750	2809	375	Filing a submission after final rejection (37 CFR § 1.129(a))	
1810	750	2810	375	For each additional invention to be examined (37 CFR § 1.129(b))	
1801	750	2801	375	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid

SUBTOTAL (3) ($)0

SUBMITTED BY			
Name (Print/Type)	Barry L. Davison	Registration No. Attorney/Agent)	47,309
Signature		Date	July 11, 2003

22504

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Docket Number 49321-97
EXPRESS MAIL NO. EL284452776US

INHIBITION OF FLAVIVIRUS REPLICATION BY INHIBITORS OF *SRC*-FAMILY KINASES

FIELD OF THE INVENTION

The present invention relates to the novel use of inhibitors of *src*-family kinases for inhibition of flavivirus infection.

BACKGROUND

The prototypical flavivirus, yellow fever virus (YFV), was first isolated in 1927. Since that time, the membership of the genus *Flavivirus* has grown to over 70 known viruses, of which more than half are associated with human disease. T hese include West Nile virus (WNV), Japanese encephalitis virus (JEV), St Louis encephalitis (SLE), and Dengue (DEN). The majority of the flaviviruses are vector borne, with approximately 50% transmitted by mosquitoes, and 30% carried by ticks. The remaining 20% are classified as "non-vector," which are transmitted by an as yet unidentified vector or zoonotically from rodents or bats (for extensive review, see (1)). The general transmission cycle of the vector borne viruses involves the acquisition of the virus by the arthropod through feeding on an infected host (usually birds, small mammals, or primates). he virus replicates in the insect host, which in turn can infect an immunologically naïve bird (or small mammal or primate, depending on the virus).

In the case of WNV, human infection through the bite of an infected mosquito results in fever in 20 percent of cases, and 1 in 150 infections result in neurological disease. The greatest risk factor for neurological disease following infection appears to be advanced age. Treatment for WNV is supportive, there being neither a widely approved therapy available, nor a vaccine approved for use in humans, although a vaccine consisting of formalin-inactivated WNV is approved for veterinary use in horses.

The flaviviruses have been sub-classified on the basis of antigenic relatedness, or more recently, on sequence similarity. Sequence information has been used to classify the viruses into 14 clades, which correlate closely with the previous antigenic classifications (2).

Epidemiology and pathology. A large number of flaviviruses are associated with human disease, and the epidemiology and pathology of three of these, West Nile virus, Dengue virus, and Japanese Encephalitis virus, are briefly summarized here.

West Nile Virus (WNV) is a mosquito borne pathogen associated with fever and encephalitis. It was first identified in Uganda in 1937 (3). Although outbreaks of WNV since its discovery had been sporadic and associated with mild illness, the frequency and severity of WNV disease, in horses as well as in humans, has increased since the mid 1990s (4). Outbreaks have occurred in Romania (1996), Morocco (1996), Tunisia (1997), Italy (1998), Russia (1999), Israel (1999 and 2000) and the U.S. (1999, and each summer since). The outbreak in New York in 1999 appears to mark the beginning of the spread of WNV throughout the U.S. In 1999, there were a total of 62 reported human cases isolated to the state of New York, 59 of which required hospitalization. In 2000, there were 21 cases in three states, increasing to 66 cases in ten states in 2001. At the time of this writing in 2002, the CDC reports 3,580 laboratory-positive human cases over 38 states (5). If the spread of the virus is measured by the presence of infected birds or mosquitoes, the geographic extent is even greater, encompassing 43 states. It is expected that the summer of 2003 will see the virus spread further. Transmission involves cyclic transfer from mosquitoes of the genus *Culex* to birds and back. Humans and horses are dead-end hosts (6).

Approximately 20% of individuals infected with WNV develop fever, as estimated by a serological survey conducted just after the 1999 New York outbreak (7). This study estimates that the total number of infections during this period was 8,200 of which 62 were reported. The fever is sometimes accompanied by weakness, nausea, headache, myalgia, arthralgia, and rash. About 1 in 150 infections results in neurological disease such as encephalitis or meningitis (7, 8). Of the 59 WNV patients hospitalized in New York in 1999, 54 were diagnosed with encephalitis or meningitis; 12% of these hospitalized patients later died. In 2002, 211 of the reported cases

resulted in death (approximately a 6% fatality rate). The greatest risk factor for death is advanced age (9). There are currently no approved antiviral therapies for WNV; treatment is supportive.

Dengue Virus (DEN). Dengue virus infects approximately 100 million people a year. It is endemic in virtually all the tropic areas of the world. There are four serotypes of DEN (Dengue type 1-4). All are spread primarily by the mosquito *Aedes Aegypti,* which lives in close proximity to humans (*i.e.* a "domestic" mosquito). Unlike the case for most flaviviruses, humans are a natural host for dengue, and can produce high enough titers in the blood to continue the transmission cycle (1, 10, 11).

DEN infection may result in one of several syndromes (12). Dengue infection is characterized by fever, headache and rash. A more severe form, Dengue hemorrhagic fever (DHF) may include increased vascular permeability and leakage of plasma from blood vessels into tissue. Mild hemorrhage may also occur. DHF is graded on a scale of I through IV. Grade II includes greater bleeding (gum, nose, GI tract), while grades III and IV feature increased vascular leakage, accompanied by loss of blood pressure and shock. Grades III and IV are also known as Dengue shock syndrome. DHF is more likely to occur when DEN infection is followed by a second infection of a different serotype. This may be due to the presence of circulating antibody that reacts with, but does not neutralize, the second infecting strain. The presence of these antibodies allows antibody-dependent enhancement of infection of macrophages, which take up antibody-bound DEN via their Fc receptors. It is postulated that macrophage infection results in increased T cell activation and cytokine production, leading to severe immunopathology (13). This model does not explain, however, the relative rarity of DHF even in patients experiencing a second DEN infection, or the occasional appearance of DHF during primary DEN infection. Other theories of DHF pathogenesis include the possibility of virulence factors present only in specific DEN strains or "quasispecies," or the possibility of an autoimmune response elicited by the similarity of DEN antigens to various human clotting factors (14, 15, 16).

Japanese Encephalitis Virus (JEV). JEV is endemic in much of southeast Asia, ranging from Japan and Korea at its northern range, to India in the west, and Indochina and Indonesia to the south. Sporadic cases have also been reported as far south as Papua New Guinea and Australia. Annually, there are approximately 35,000 cases and 10,000 deaths, and these figures may underestimate the true toll of the disease due to incomplete surveillance and reporting. JEV is a member of an antigenic complex and clade that also include WNV. It is spread primarily by the mosquito *Culex tritaeniorhynchus*, cycling through its natural viremic hosts, pigs and birds.

Most JEV infections are sub-clinical, with only 1 in 250 infections resulting in symptoms. The primary clinical manifestation is encephalitis. After a 5-15 day incubation period, symptoms begin with headache, fever, and gastrointestinal problems. These may be followed by irritability, nausea, and diarrhea with decline to generalized weakness, stupor, or coma. In children, seizures are common. 5-30% of cases are fatal.

There is no specific treatment for JEV, other than supportive care. However, vaccines do exist for JEV. These include a formalin inactivated vaccine, as well as a live attenuated strain. The inactivated version has been used widely in Japan and China since the 1960s. It is also licensed for use in the U.S. and Europe for those traveling to areas in which JEV is endemic. The attenuated virus has also seen wide use in China. Both vaccines (when delivered with appropriate booster regimens) have shown efficacies greater than 90% (1, 17).

Flavivirus replication. The flaviviruses are small enveloped viruses that contain a single, positive-sense RNA genome of approximately 11 kilobases (kb). The RNA is capped at its 5' end, but not 3' polyadenylated. The RNA encodes a single large open reading frame (ORF) that is processed into 10 subunits that comprise the structural components of the virion and the viral replication complex (18). The flaviviruses all possess a common organization to the coding sequence of the genome. The structural subunits are located at the 5' end. These include the core (C), membrane (prM/M), and envelope (E) proteins. These are followed by the non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. NS2B and NS3 function as the serine protease that is responsible for processing much of the viral polyprotein. NS5, the

most highly conserved of the flavivirus proteins, acts as the RNA dependent RNA polymerase necessary for viral replication, and may also function as a methyltransferase that provides the genomic 5' cap. The other members of the non-structural group are largely hydrophobic and of unknown function (18).

Flavivirus infection of the host cell begins via attachment of the E protein to a cellular receptor. Definitive identification of a receptor for any of the flavivirus species is still absent, but glycosaminoglycans appear to be involved in the initial attachment (19). Entry of the virus into the host cell probably occurs by receptor mediated endocytosis, followed by low-pH dependent fusion of the virion with the endosome membrane, releasing the nucleocapsid and genomic RNA into the cytoplasm (18, 20).

Translation of the RNA by the host cell follows, and the polyprotein is cleaved into its constituent subunits by a combination of a host cell ER resident protease and the NS2B/NS3 virally encoded serine protease. Replication of the genomic RNA occurs through a negative sense intermediate, and can be detected as early as three hours after infection in the case of YFV. Flavivirus infection induces a proliferation of ER membranes in the host cell and the formation of "smooth membrane structures"- groups of vesicle-like structures in the ER lumen. The smooth membrane structures co-localize with double stranded RNA (presumably the replicative intermediate), as well as NS1 and NS3, and are believed to be the sites of RNA replication. NS2B and NS3, the constituents of the viral protease, localize to an adjacent region of induced membranes (dubbed "convoluted membranes"), suggesting that polyprotein processing and NA replication are spatially separated within the infected cell (21).

Assembly and release of virions largely remains a black box. Cis-acting packaging signals in the RNA have not been identified, although the viral nucleocapsid protein C has been shown to interact with the 5' and 3' ends of the genome (22). The envelope is most likely acquired by budding of the nucleocapsid precursor into the ER. At a later point in virus maturation, the prM protein is cleaved into the mature form (M) by the cellular protease furin (23). It is currently believed that prM functions to prevent the E protein from undergoing the

low pH dependent conformational change while in the cell. In agreement with this hypothesis, prevention of prM cleavage results in the release of virus particles that are less infectious than wild-type (24).

Infection of the host is thought to begin in the Langerhans cells of the skin following the bite of a carrier arthropod. Viral replication continues in the regional tissue and lymph nodes, which results in the dissemination of the virus into the bloodstream. Replication then proceeds at several sites, including connective tissue, smooth muscle, liver and spleen. Neural invasion appears to occur through the olfactory epithelium in experimentally infected rodents. It is unclear if this is the primary route used by virus to gain access to the CNS in infected humans (25, 26).

Treatment and vaccine development. Treatment for most flavivirus infections resulting in disease is supportive (*i.e.* fluid management, mechanical ventilation, transfusion in case of severe hemorrhage, etc.). Recent reports show efficacy of ribavirin and interferon-α2b in WNV infection, although controlled clinical trials have not been completed (8). Preventive vaccines exist for YFV and JEV, both based on live, attenuated strains. Similar strategies as well as the construction of chimeric viruses based on the backbones of approved flavivirus vaccines are being used to develop vaccines against WNV, Dengue, and others (27).

There is a pronounced need in the art for novel therapeutic methods and compositions having utility for preventing or inhibiting flavivirus infection, and for treatment and/or prevention of conditions related to flavivirus infection.

SUMMARY OF THE INVENTION

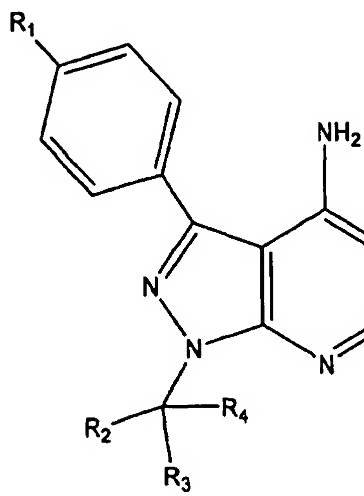
The present invention provides methods and compositions having utility for preventing or inhibiting flavivirus infection, and for treatment and/or prevention of conditions related to flavivirus infection. Specifically, inhibitors of *src* family kinases have been shown to have said utilities. Preferably, the inventive methods and compositions are directed to inhibition of the *src* family kinase c-yes. Preferably, the flavivirus is selected from the group consisting of West Nile

virus (WNV), Japanese encephalitis virus (JEV), St. Louis encephalitis (SLE), and Dengue (DEN). Preferably, the flavivirus is WNV. Preferably, the *src* family kinase inhibitor is PP2 (4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; Calbiochem). Preferably, the *src* family kinase inhibitor is 2-oxo-3-(4,5,6,7-tetrahydro-1*H*-indol-2-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid dimethylamide (SU6656).

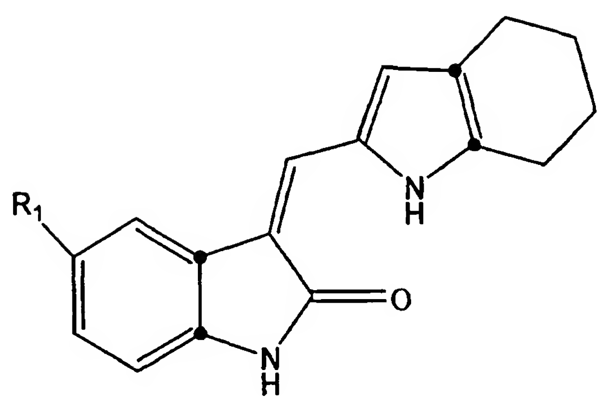
Further embodiments of the present invention provide screening assays for identification of agents having therapeutic utility for preventing or inhibiting flavivirus infection, and for treatment and/or prevention of conditions related to flavivirus infection.

Particular embodiments, the present invention provide a method for the treatment of flavivirus infection and related conditions, comprising administration, to a subject in need thereof, of a therapeutically effective amount of an inhibitor of a *src* family kinase, whereby at least one of flavivirus infection or related conditions are diminished relative to non-treated subjects. Preferably, the flavivirus is selected from the group consisting of West Nile virus (WNV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), Dengue fever virus (DEN), and combinations thereof. Preferably, the *src* family kinase is c-yes kinase.

Preferably, the inhibitor is selected from the group consisting of *src* family kinase-specific antisense RNA, *src* family kinase-specific siRNA, and a small molecule inhibitor of a *src* family kinase. Preferably, the *src* family kinase is c-yes kinase. Preferably, the inhibitor is compound having the structure of Formula I, or Formula II, or salts thereof:



Formula I

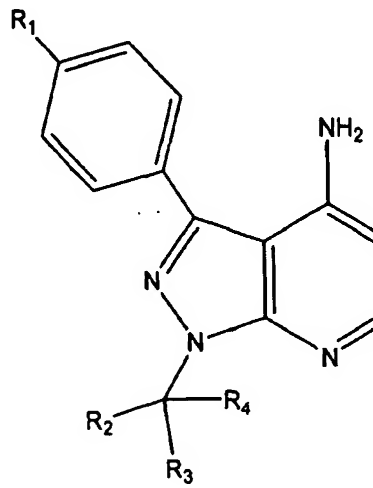


Formula II

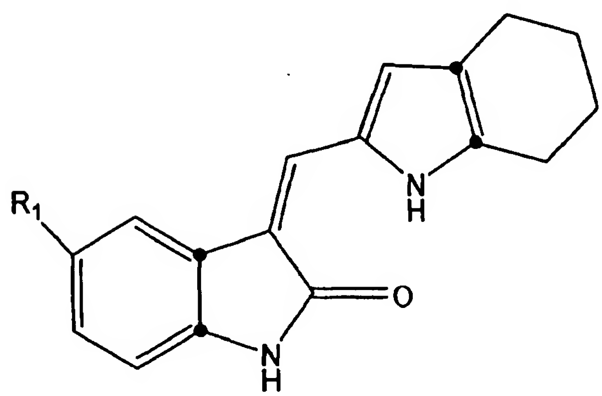
Preferably, for Formula 1, R_1 is halogen, and R_2, R_3 and R_4 are independently a C1-C3 straight or branched alkyl. Preferably, Formula II, R_1 is $-SO_2N(CH_3)_2$, or $-SO_2NH_2$. Preferably, the inhibitor is 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2). Preferably, the inhibitor is 2-oxo-3-(4,5,6,7-tetrahydro-1*H*-indol-2-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid dimethylamide (SU6656).

Additional embodiments provide pharmaceutical compositions having utility for the treatment of flavivirus infection and related conditions, comprising a *src* family kinase inhibitor selected from the group consisting of *src* family kinase-specific antisense RNA, *src* family kinase-specific siRNA, and a small molecule inhibitor of a *src* family kinase, along with a

pharmaceutically acceptable carrier or excipient. Preferably, the *src* family kinase is c-yes kinase. Preferably, the inhibitor is compound having the structure of Formula I, Formula II, or salts thereof:



Formula I



Formula II

Preferably, for Formula I, R_1 is halogen, and wherein R_2, R_3 and R_4 are independently a C1-C3 straight or branched alkyl. Preferably, for Formula II, R_1 is $-SO_2N(CH_3)_2$, or $-SO_2NH_2$.

Preferably, the inhibitor is 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2). Preferably, the inhibitor is 2-oxo-3-(4,5,6,7-tetrahydro-1*H*-indol-2-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid dimethylamide (SU6656).

Yet further embodiments provide a method for identification of agents having potential therapeutic utility for the treatment of flavivirus infection and related conditions, comprising:

obtaining cells suitable to support a flavivirus infection; infecting the cells with flavivirus; contacting the infected cells with an agent that inhibits a src family kinase; and determining whether the flavivirus infection is diminished relative to control infected cells not contacted by the agent, whereby potential therapeutic agents are, at least in part, identified. Preferably, the flavivirus is selected from the group consisting of West Nile virus (WNV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), Dengue fever virus (DEN), and combinations thereof. Preferably, the *src* family kinase is c-yes kinase. Preferably, the cell suitable to support flavivirus infection are selected from the group consisting of primary human hepatocellular carcinoma derived cells or cell-lines derived therefrom, Huh 7 cells, neuroblastoma cells or cell-lines derived therefrom, SKN-MC cells, and combinations thereof. Preferably, infection precedes contacting of the cells with the agent. Preferably, infection is subsequent to contacting of the cells with the agent.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows that the scr family kinase inhibitor PP2 (4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) inhibits accumulation of infectious WNV in cell culture supernatants (see EXAMPLE I, below).

Figure 1B shows the effect of PP2 in inhibiting accumulation of infectious virus within infected cells. SKN-MC cells were infected with WNV as above. Infectious virus in soluble lysate was measured by plaque assay on Vero cells. Samples were collected 4 hours post addition of fresh PP2 (gray bars) and 24 hours post addition of fresh PP2 (black bars).

Figure 1C shows that levels of WNV RNA within infected SKN-MC cells did not change with PP2 addition. WNV RNA quantities were normalized to β-actin values.

Figure 2A shows reduction of c-yes mRNA in transfected Huh7 cells in response to c-yes specific siRNA. 1μg total cellular RNA was used for quantitative RT-PCR with c-yes specific primers.

Figure 2B shows WNV in siRNA treated cultures. Culture supernatant from Huh7 cells transfected with c-yes specific siRNAs (above) were harvested 24 hpi and virus measured by plaque assay on Vero cells.

Figure 3 shows K-means clusters corresponding to 238 gene with changes at any one time point post-WNV infection.

Figure 4 shows a classification by function of genes up-regulated at 15 h post-infection (hpi) in West Nile Virus cells (strain NY1999).

Figure 5 shows plots of relative gene expression, for exemplary up-regulated genes, at various hour post infection with Japanese encephalitis virus (JEV; diamond symbols), West Nile virus (WNV; square symbols) and mock infection (triangles).

Figure 6 shows the effect of tyrosine kinase inhibitors on WNV (NY1999) infection in a human hepatocellular carcinoma-derived cell line (Huh7 cells).

Figure 7 shows the effect of *src* family tyrosine kinase (SFK) inhibitors on WNV (NY1999) infection in SKN-MC neuroblastoma cells.

Figure 8 shows the amount of intracellular infectious WNV after SFK inhibitor treatment in SKN-MC cells.

Figure 9 shows the amount of intracellular WNV RNA following PP2 treatment in SKN-MC cells.

Figure 10 shows that C-yes-specific siRNA inhibits WNV replication in Huh7 cells.

DETAILED DESCRIPTION OF THE INVENTION

Overview

As stated herein above, there is currently no approved therapy to combat WNV, or other flavivirus infections. The present invention encompasses, *inter alia*, use of inhibitors of *Src*-family kinases to treat and/or prevent flavivirus infection and related conditions, based on the discovery that *Src*-family kinases, and particulary the c-yes kinase are novel therapeutic intervention targets for flavivirus infection

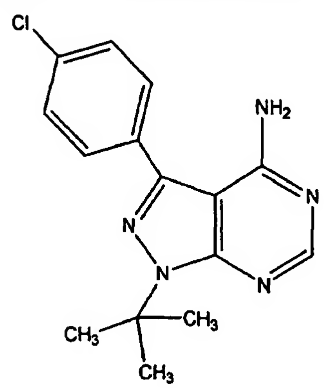
Additional embodiments provide therapeutic compositions useful to treat and/or prevent flavivirus infection and related conditions.

Further embodiments provide screening assays for the identification of inhibitors of *Src*-family kinases that inhibit flavivirus infection and related conditions.

Preferably, the flavivirus is selected from the group consisting of West Nile virus (WNV), Japanese encephalitis virus (JEV), St. Louis encephalitis (SLE), and Dengue (DEN).

Preferably, the flavivirus is WNV.

Preferably, the scr family kinase inhibitor is PP2 (see Formula I, below) (4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) (Calbiochem; catalog no. 529573), or a suitable salt or derivative thereof.



Formula I

DEFINITIONS

The term "WNV" refers to West Nile virus, a flavivirus.

The term "JEV: refers to Japanese encephalitis virus, a flavivirus.

The term "SLE" refers to St. Louis encephalitis, a flavivirus.

The term "DEN" refers to Dengue, a flavivirus.

The term "PP2" (Formula I, above) refers to 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (*e.g.*, Calbiochem; catalog no. 529573).

The term "Huh7" refers to the art-recognized human hepatocellular carcinoma derived cell line.

The term "SKN-MC" refers to art-recognized neuroblastoma cells.

The term "SU6656" refers to (2-oxo-3-(4,5,6,7-tetrahydro-1*H*-indol-2-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid dimethylamide) (*see* Blake et al., *Mol. Cell. Biol.* 20:9018-9027, Dec. 2000).

The term "SU6657" refers to (2-oxo-3-(4,5,6,7-tetrahydro-1*H*-indol-2-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid amide) (*Id*).

METHODS AND COMPOSITIONS FOR INHIBITION OF FLAVIVIRUS INFECTION

According one aspect of the present invention, human hepatocellular carcinoma derived cell line Huh7 supports replication of flaviviruses, including West Nile virus (WNV), Japanese encephalitis virus (JEV), yellow fever virus (YFV) and Dengue fever virus (DEN). The Huh7 cell line was used as a host cell model for particular embodiments described herein. Additionally, SKN-MC neuroblastoma cells were used for particular embodiments described herein.

Gene microarrays were used to define the general host cell response to flavivirus infection. Specifically, gene microarrays comprising representations of 8,100 human genes were used to compare host cell gene expression following infection with WNV, JEV, YFV and DEN. For WNV, K-means clusters corresponding to 238 genes having changes in expression at any one time point post-WNV infection are shown in Figure 3.

Likewise, microarray results with JEV showed greater than 100 host genes whose expression was increased by between 2- and 10-fold at 15 h post-infection (hpi). Additionally, quantitative RT-PCR (Taqman) was used to show that several of these genes are also up-regulated in Huh7 cells during WNV infection (*e.g.*, at 15 hpi).

Figure 4 shows a classification by function of genes up-regulated at 15 h post-infection (hpi) in West Nile Virus. Analysis of the up-regulated genes revealed that they include components of a signal transduction cascade that signals through a member of the src-family tyrosine kinase family (Figures 4 and 5).

Figure 5 shows plots of the observed relative gene expression, for exemplary up-regulated genes, at various times (hours) post-infection with Japanese encephalitis virus (JEV; diamond symbols), West Nile virus (WNV; square symbols) and mock infection (triangles).

Significantly, for example, as shown in EXAMPLE 1 below, the addition of a specific inhibitor of src-family kinases, to Huh7 cells at the time of WNV infection resulted in a dose-dependent reduction of virus recovered from the culture supernatant, as measured by plaque

assay and quantitative RT-PCR of viral RNA (up to 90%, as compared to control samples). Inhibition of src-family kinase, however, did not result in a corresponding reduction of viral RNA found within the infected cells, indicating that this pathway (*i.e.*, src family kinases, and related signal transduction) plays a role in viral assembly or egress from the host cell.

Likewise, src-family kinases can be inhibited by antisense, ribozymes, and siRNA.

<u>siRNA</u>

The invention, in particular aspects, contemplates introduction of RNA with partial or fully double-stranded character into the cell or into the extracellular environment. Inhibition is specific to the *src* family kinase expression in that a nucleotide sequence from a portion of the target *src* family kinase gene is chosen to produce inhibitory RNA. This process is effective in producing inhibition (partial or complete), and is kinase gene-specific. In particular embodiments, the target cell containing the target *src* family kinase gene may be a human cell subject to infection by flaviviruses, or transformed cells (*e.g.*, hepatocellular carcinoma cells or cell-lines derived therefrom, neuroblastoma cells or cell-lines derived therefrom). Methods of preparing and using RNAi are generally disclosed in U.S. Patent 6,506,559, incorporated herein by reference.

The RNA may comprise one or more strands of polymerized ribonucleotide, and may include modifications to either the phosphate-sugar backbone or the nucleoside. For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. Modifications in RNA structure may be tailored to allow specific genetic inhibition while avoiding a general panic response in some organisms which is generated by dsRNA. Likewise, bases may be modified to block the activity of adenosine deaminase. RNA may be produced enzymatically or by partial/total organic synthesis, any modified ribonucleotide can be introduced by *in vitro* enzymatic or organic synthesis.

The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside

or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses of double-stranded material may yield more effective inhibition. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition. NA containing a nucleotide sequence identical to a portion of the *src* family kinase target gene is preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Sequence identity may be optimized by alignment algorithms known in the art and calculating the percent difference between the nucleotide sequences. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript.

RNA may be synthesized either *in vivo* or *in vitro*. Endogenous RNA polymerase of the cell may mediate transcription in vivo, or cloned RNA polymerase can be used for transcription *in vivo* or *in vitro*. For transcription from a transgene in vivo or an expression construct, a regulatory region may be used to transcribe the RNA strand (or strands).

For RNAi, the RNA may be directly introduced into the cell (*i.e.*, intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing RNA. Methods for oral introduction include direct mixing of RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express an RNA, then fed to the organism to be affected. Physical methods of introducing nucleic acids include injection directly into the cell or extracellular injection into the organism of an RNA solution.

Inhibition of gene expression refers to the absence (or observable decrease) in the level of protein and/or mRNA product from a *src* family kinase target gene. Specificity refers to the ability to inhibit the target gene without manifest effects on other genes of the cell. The consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism or by biochemical techniques such as RNA solution hybridization, nuclease

protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays, fluorescence activated cell analysis (FACS), and flavivirus viral infection as described herein. For RNA-mediated inhibition in a cell line or whole organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Many such reporter genes are known in the art.

For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. Modifications in RNA structure may be tailored to allow specific genetic inhibition while avoiding a general panic response in some organisms which is generated by dsRNA. Likewise, bases may be modified to block the activity of adenosine deaminase. RNA may be produced enzymatically or by partial/total organic synthesis, any modified ribonucleotide can be introduced by *in vitro* enzymatic or organic synthesis.

RNA containing a nucleotide sequences identical to a portion of the *src* family kinase target gene are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence may be effective for inhibition. Thus, sequence identity may optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (*e.g.*, University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of the *src* family kinase target gene is preferred. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the *src* family kinase target gene transcript (*e.g.*, 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C. or 70°C. hybridization for 12-16 hours; followed by washing). The length of the identical nucleotide sequences may be at least 25, 50, 100, 200, 300 or 400 bases.

A 100% sequence identity between the RNA and the *src* family kinase target gene is not required to practice the present invention. Thus the methods have the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence.

src family kinase RNAi may be synthesized by art-recognized methods either *in vivo* or *in vitro*. Endogenous RNA polymerase of the cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription in vivo or in vitro. For transcription from a transgene *in vivo* or an expression construct, a regulatory region (e.g., promoter, enhancer, silencer, splice donor and acceptor, polyadenylation) may be used to transcribe the RNA strand (or strands). Inhibition may be targeted by specific transcription in an organ, tissue, or cell type; stimulation of an environmental condition (*e.g.*, infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. The RNA strands may or may not be polyadenylated; the RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus.

RNA may be chemically or enzymatically synthesized by manual or automated reactions. The RNA may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (e.g., T3, T7, SP6). The use and production of an expression construct are known in the art (for example, WO 97/32016; U.S. Pat. Nos. 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693; and the references cited therein). If synthesized chemically or by in vitro enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For example, RNA can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography, or a combination thereof. Alternatively, the RNA may be used with no or a minimum of purification to avoid losses due to sample processing. The RNA may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to promote annealing, and/or stabilization of the duplex strands.

RNA may be directly introduced into the cell (*i.e.*, intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced

orally, or may be introduced by bathing an organism in a solution containing the RNA. Methods for oral introduction include direct mixing of the RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express the RNA, then fed to the organism to be affected. For example, the RNA may be sprayed onto a plant or a plant may be genetically engineered to express the RNA in an amount sufficient to kill some or all of a pathogen known to infect the plant. Physical methods of introducing nucleic acids, for example, injection directly into the cell or extracellular injection into the organism, may also be used. Vascular or extravascular circulation, the blood or lymph system, and the cerebrospinal fluid are sites where the RNA may be introduced. A transgenic organism that expresses RNA from a recombinant construct may be produced by introducing the construct into a zygote, an embryonic stem cell, or another multipotent cell derived from the appropriate organism.

Physical methods of introducing nucleic acids include injection of a solution containing the RNA, bombardment by particles covered by the RNA, soaking the cell or organism in a solution of the RNA, or electroporation of cell membranes in the presence of the RNA. A viral construct packaged into a viral particle would accomplish both efficient introduction of an expression construct into the cell and transcription of RNA encoded by the expression construct. Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical-mediated transport, such as calcium phosphate, and the like. Thus the RNA may be introduced along with components that perform one or more of the following activities: enhance RNA uptake by the cell, promote annealing of the duplex strands, stabilize the annealed strands, or other-wise increase inhibition of the target gene.

The RNAi may be used alone or as a component of a kit having at least one of the reagents necessary to carry out the in vitro or in vivo introduction of RNA to test samples or subjects. Preferred components are the dsRNA and a vehicle that promotes introduction of the dsRNA. Such a kit may also include instructions to allow a user of the kit to practice the invention.

Suitable injection mixes are constructed so animals receive an average of 0.5 x 10^6 to 1.0 x 10^6 molecules of RNA. For comparisons of sense, antisense, and dsRNA activities, injections are compared with equal masses of RNA (*i.e.*, dsRNA at half the molar concentration of the single strands). Numbers of molecules injected per adult are given as rough approximations based on concentration of RNA in the injected material (estimated from ethidium bromide staining) and injection volume (estimated from visible displacement at the site of injection). A variability of several-fold in injection volume between individual animals is possible.

EXAMPLE 1

(*src* family kinase inhibitors resulted in a substantial decrease of infectious WNV)

DNA Microarray analysis

Methods. Viral stocks for all microarray infections were grown on Vero cells. Viral titers were determined by limiting dilution on Veros. In order to avoid effects from cytokines present in viral stocks, the virus was concentrated and purified before infection of target cells. Approximately 28 ml of stock was underlayed with 7 ml of 20% sorbitol cushion and spun for 1.5 h at 25,000 rpm (113,000 × g) and 20°C in an SW28 rotor (Beckman). The virus pellet was resuspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 0.1% bovine serum albumin (BSA). Approximately 3 × 10^6 Huh7 cells were infected at a multiplicity of 10 with the concentrated virus stock. At 0.5h, 2h, 5h, 10h, 15h, 20h and 26h post-infection, duplicate plates were collected and RNA isolated by TRIzol reagent (Invitrogen) according to manufacturer's instructions. Labled cDNA was generated from the total RNA and hybridized to cDNA spotted arrays. The spotted arrays contained cDNAs representing almost 8400 genes. Approximately 200 genes were identified as having increased expression in both JEV and WNV infected Huh7 cells at the 15 hour time point.

Confirmation of microarray results. Quantitative real-time RT-PCR analysis was performed on RNA samples harvested from independent infections of Huh7 cells with JEV or WNV, to confirm the results of the initial microarray experiment. As shown in Figures 5A or

5B, four of the genes reported to be upregulated at 15 h by the spotted array experiment are confirmed by RT-PCR analysis. RT-PCR was performed using Omniscript reverse transcriptase (Qiagen) and random hexamer primers, followed by PCR in an ABI Prism 7700 Sequence Detector using gene specific primers in the presence of SYBR green (Applied Biosystems). Interestingly, and in agreement with the array results, the upregulation seems to be a transient event that occurs at approximately 15 h post infection, but is absent by 24 h post infection.

Addition of the *src* family kinase inhibitor PP2 (4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; *e.g.*, Calbiochem; catalog no. 529573) at 20 μM to WNV infected SKN-MC neuroblastoma cells resulted in a 30-fold decrease of infectious WNV in the supernatant 22 hours post-infection (20 h post-PP2 addition) (Figure 1A, and Figure 7). Similar results were obtained using Huh7 cells (Figure 6).

Specifically, Figure 1A shows that PP2 inhibits accumulation of infectious WNV in cell culture supernatants. SKN-MC neutoblastoma cells were infected with WNV at a multiplicity of 5. Two (2) hours post-infection (hpi, cells were treated with PP2 to a final concentration of 20 μM, or DMSO only as a control. Culture supernatant was harvested 20 hours post-infection, and virus was measured by plaque assay on Vero cells. Likewise, Figure 7 shows the observed effect of *src* family tyrosine kinase (SFK) inhibitors on WNV (NY1999) infection in SKN-MC neuroblastoma cells.

Figure 6 shows the observed effect of tyrosine kinase inhibitors on WNV (NY1999) infection in a human hepatocellular carcinoma-derived cell line (Huh7 cells).

Additionally, infectious virus *within* infected cell lysates was decreased almost 10^6-fold four hours post-addition of PP2 (Figure 1B, gray bars; and Figure 8).

Figure 1B shows the effect of PP2 in inhibiting accumulation of infectious virus within infected cells. Specifically, SKN-MC cells were infected with WNV as above. At 2 hpi, cells were treated with PP2 at the indicated final concentrations. At 20 hpi, cells were re-fed with media containing fresh PP2 at the same concentration. Cells were washed 3-times with phosphate buffered saline (PBS), resuspended in 250 μl PBS, and then lysed by 3 successive

freeze/thaws. Lysates were centrifuged at 13,000 x g for 5 minutes, followed by collection of the supernatant. Infectious virus in soluble lysate was measured by plaque assay on Vero cells. Samples were collected 4-hours post-addition of fresh PP2 (gray bars) and 24 hours post addition of fresh PP2 (black bars). 24-hours post-addition of PP2, virus within the infected cell was approximately 500-fold lower, indicating some recovery from the effect of the drug (Fig. 1B, black bars). Figure 8 shows the amount of intracellular infectious WNV after SFK inhibitor treatment in SKN-MC cells.

Quantification of viral RNA within infected cells (4 hours post PP2 addition) showed no significant difference between treated or untreated cells (Figure 1C; and Figure 9), suggesting that the effect of the inhibitor is exerted at a post-viral RNA replication stage.

Figure 1C shows that levels of WNV RNA within infected SKN-MC cells did not change with the addition of PP2. Specifically, cells were prepared as described above in relation to Figure 1B. Four hours post fresh PP2 addition, RNA was isolated from 200 μl of cell lysate by Trizol reagent (Invitrogen), and 1 μg total RNA was used in a quantitative RT-PCR reaction (Taqman) using WNV-specific primer and probe set and standards. An additional RT-PCR was performed using β-actin-specific primers and probe, for an internal control. WNV RNA quantities were normalized to the β-actin values. Figure 9 shows the amount of intracellular WNV RNA following PP2 treatment in SKN-MC cells.

Additionally, in Huh7 hepatocellular carcinoma cells, transfection of siRNA designed to inhibit production of c-yes, but not a control siRNA (lamin A/C) resulted in an approximately 8-fold reduction of the amount of WNV in the culture supernatant (Figures 2A and B; and Figure 10).

Specifically, Figure 2A shows the observed reduction of c-yes mRNA in response to c-yes-specific siRNA. Huh7 cells were transfected with either of c-yes-specific siRNAs 214 or 316, or a control siRNA directed against Lamins A and C. Transfection of 300,000 cells was carried out with 2 μl of a 20 μM siRNA (Dharmacon) and 1 μl Oligofectamine (Invitrogen) according to the manufacturer's protocol. Cells were transfected 24 hours post-transfection, and

cells and supernatant harvested at 24 hpi. 1 μg total cellular RNA was used for quantitative RT-PCR with c-yes-specific primers.

Figure 2B shows the measured levels of WNV in the siRNA treated cultures. Culture supernatant from Huh7 cells transfected with c-yes specific siRNAs (above) were harvested 24 hpi, and virus was measured by plaque assay on Vero cells.

Figure 10 shows that C-yes-specific siRNA inhibits WNV replication in Huh7 cells.

These results identify and validate c-yes as a *src*-family kinase therapeutic target for the treatment of flavivirus and related conditions, and indicates that PP2 may exert its effect on flavivirus infection, at least in part, through this target.

At 24 hours post-addition of PP2 (Figure 2B; and Figure 8), intracellular levels of WNV have recovered somewhat relative to untreated cells.

According to the present invention, inhibitors with longer effective half-lives have a longer period of WNV inhibition.

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CLAIMS

We Claim:

1. A method for the treatment of flavivirus infection and related conditions, comprising administration, to a subject in need thereof, of a therapeutically effective amount of an inhibitor of a *src* family kinase, whereby at least one of flavivirus infection or related conditions are diminished relative to non-treated subjects.

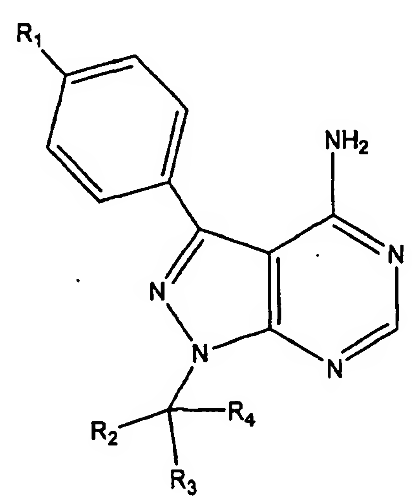
2. The method of claim 1, wherein the flavivirus is selected from the group consisting of West Nile virus (WNV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), Dengue fever virus (DEN), and combinations thereof.

3. The method of claim 1, wherein the *src* family kinase is c-yes kinase.

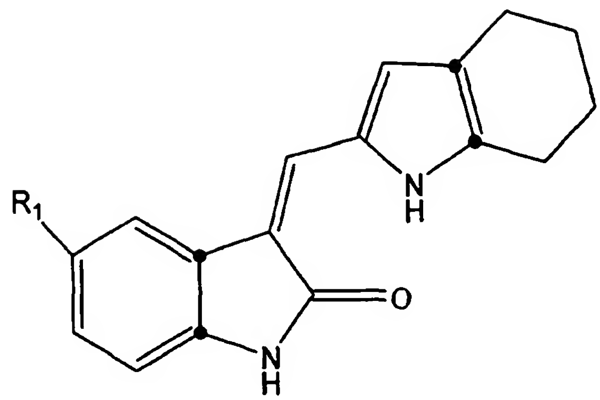
4. The method of claim 1, wherein the inhibitor is selected from the group consisting of *src* family kinase-specific antisense RNA, *src* family kinase-specific siRNA, and a small molecule inhibitor of a *src* family kinase.

5. The method of claim 4, wherein, the *src* family kinase is c-yes kinase.

6. The method of any one of the preceding claims, wherein the inhibitor is compound having the structure of Formula I, or Formula II, or salts thereof:



Formula I



Formula II

7. The method of claim 6, wherein for Formula 1, R_1 is halogen, and R_2, R_3 and R_4 are independently a C1-C3 straight or branched alkyl.

8. The method of claim 6, wherein for Formula II, R_1 is $-SO_2N(CH_3)_2$, or $-SO_2NH_2$.

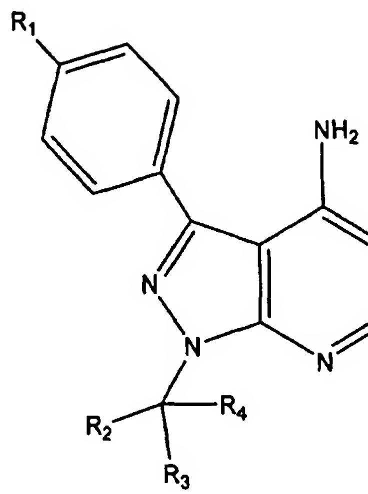
9. The method of claim 6, wherein the inhibitor is 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2).

10. The method of claim 6, wherein the inhibitor is 2-oxo-3-(4,5,6,7-tetrahydro-1*H*-indol-2-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid dimethylamide (SU6656).

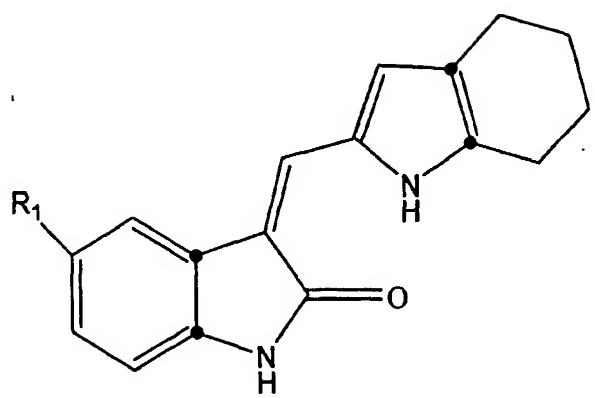
11. A pharmaceutical composition having utility for the treatment of flavivirus infection and related conditions, comprising a *src* family kinase inhibitor selected from the group consisting of *src* family kinase-specific antisense RNA, *src* family kinase-specific siRNA, and a small molecule inhibitor of a *src* family kinase, along with a pharmaceutically acceptable carrier or excipient.

12. The pharmaceutical composition of claim 11, wherein the *src* family kinase is c-yes kinase.

13. The pharmaceutical composition of claim 11, wherein the inhibitor is compound having the structure of Formula I, Formula II, or salts thereof:



Formula I



Formula II

14. The composition of claim 13, wherein, for Formula I, R_1 is halogen, and wherein R_2, R_3 and R_4 are independently a C1-C3 straight or branched alkyl.

15. The composition of claim 13, wherein for Formula II, R_1 is $-SO_2N(CH_3)_2$, or $-SO_2NH_2$.

16. The composition of claim 13, wherein the inhibitor is 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2).

17. The composition of claim 13, wherein the inhibitor is 2-oxo-3-(4,5,6,7-tetrahydro-1*H*-indol-2-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid dimethylamide (SU6656).

18. A method for identification of agents having potential therapeutic utility for the treatment of flavivirus infection and related conditions, comprising:

-obtaining cells suitable to support a flavivirus infection;

-infecting the cells with flavivirus;

-contacting the infected cells with an agent that inhibits a src family kinase; and

-determining whether the flavivirus infection is diminished relative to control infected cells not contacted by the agent, whereby potential therapeutic agents are, at least in part, identified.

19. The method of claim 18, wherein the flavivirus is selected from the group consisting of West Nile virus (WNV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), Dengue fever virus (DEN), and combinations thereof.

20. The method of claim 18, wherein the *src* family kinase is c-yes kinase.

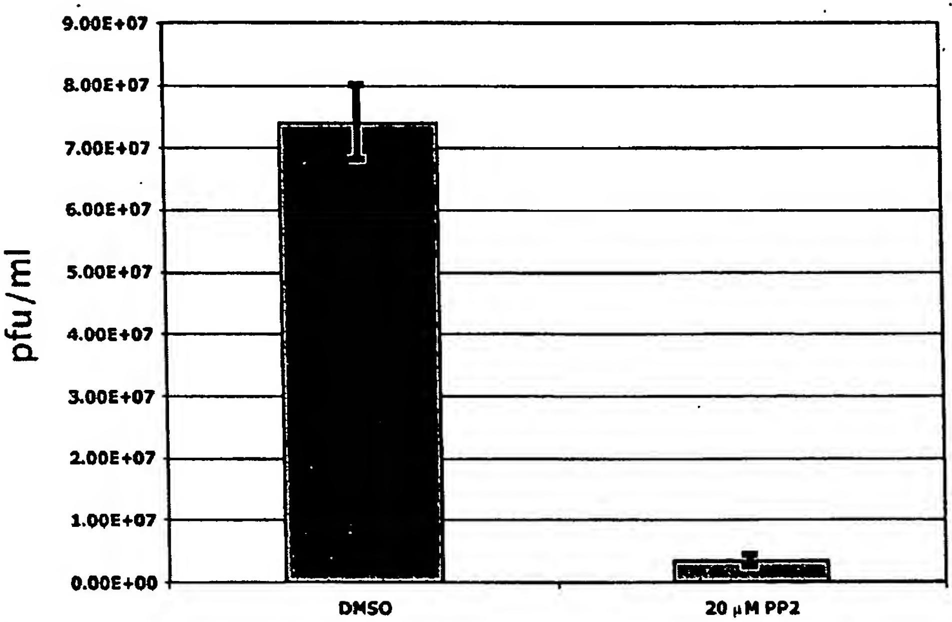
21. The method of claim 18, wherein the cell suitable to support flavivirus infection are selected from the group consisting of primary human hepatocellular carcinoma derived cells or cell-lines derived therefrom, Huh 7 cells, neuroblastoma cells or cell-lines derived therefrom, SKN-MC cells, and combinations thereof.

22. The method of claim 18, wherein infection precedes contacting of the cells with the agent.

23. The method of claim 18, wherein infection is subsequent to contacting of the cells with the agent.

ABSTRACT OF THE DISCLOSURE

Gene microarrays were used to identify cellular genes having up-regulated expression following infection with flaviviruses, including West Nile virus (WNV), Japanese encephalitis virus (JEV), yellow fever virus (YFV) and Dengue fever virus (DEN). The up-regulated genes include those corresponding to components of a signal transduction cascade that signals through a member of the src-family tyrosine kinase family. The addition of specific inhibitors of *src*-family kinases, to Huh7 cells at the time of WNV infection resulted in a substantial dose-dependent reduction of virus recovered from the culture supernatant. Particular embodiments of the present invention provide for therapeutic methods and compositions for the treatment of flavivirus infection and related conditions. Additional embodiments provide screening assays for therapeutic agents having utility for the treatment of flavivirus infection and related conditions.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7.00E+07
6.00E+07
5.00E+07
4.00E+07
3.00E+07
2.00E+07
1.00E+07
0.00E+00
pfu/ml
DMSO
20 μM PP2

Fig. 1A

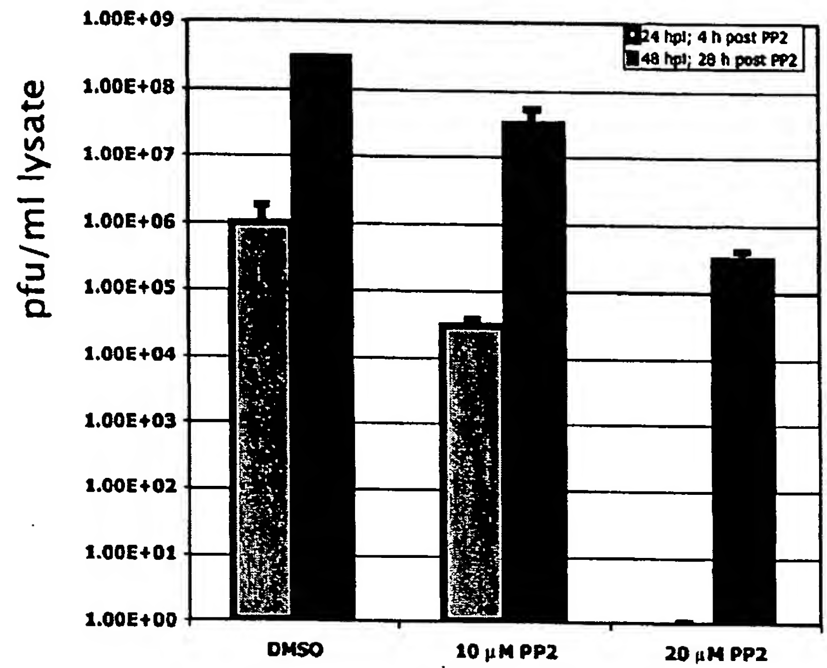

1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
pfu/ml lysate
24 hpi; 4 h post PP2
48 hpi; 28 h post PP2
DMSO
10 μM PP2
20 μM PP2

Fig. 1B

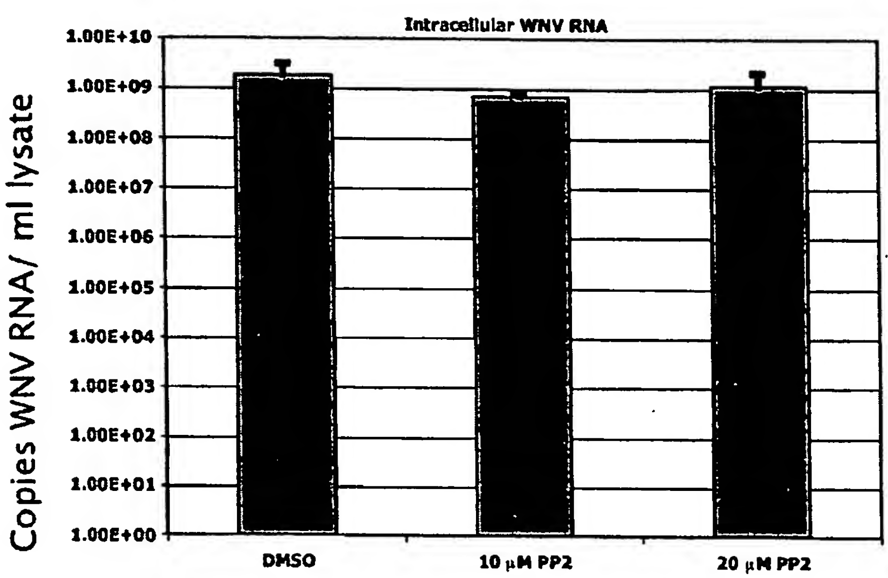
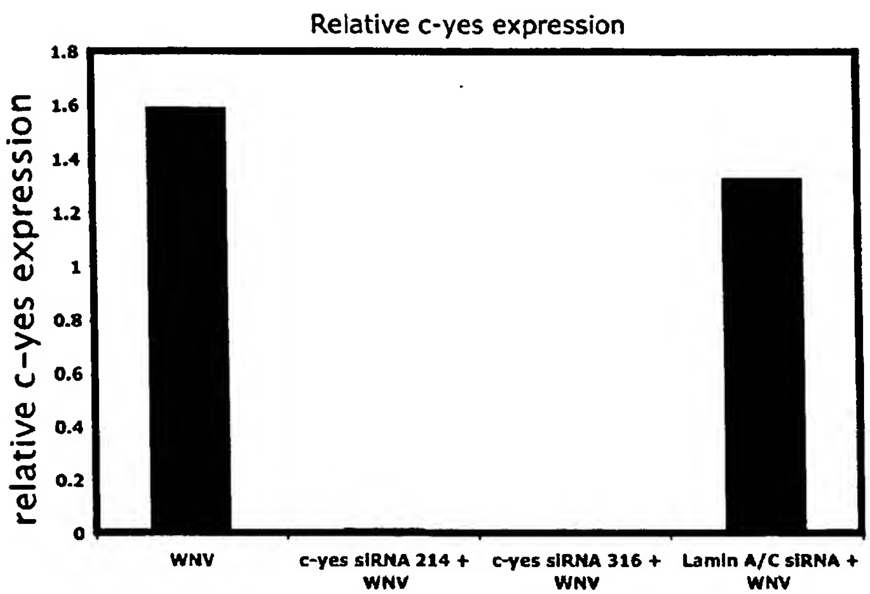

Intracellular WNV RNA
Copies WNV RNA/ ml lysate
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
DMSO
10 μM PP2
20 μM PP2

Fig. 1C



Relative c-yes expression
relative c-yes expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WNV
c-yes siRNA 214 + WNV
c-yes siRNA 316 + WNV
Lamin A/C siRNA + WNV

Fig. 2A

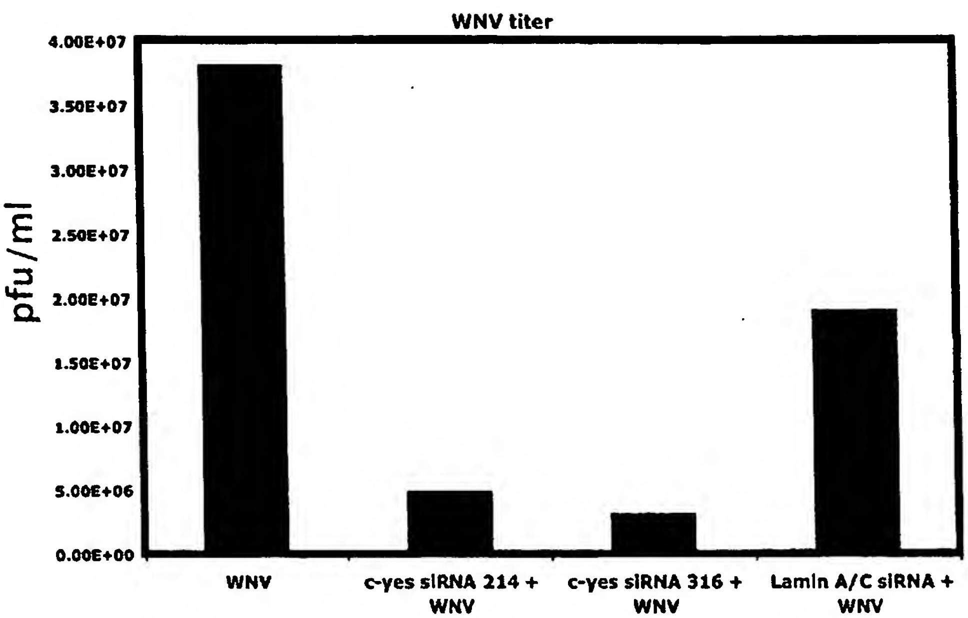

WNV titer
pfu/ml
4.00E+07
3.50E+07
3.00E+07
2.50E+07
2.00E+07
1.50E+07
1.00E+07
5.00E+06
0.00E+00
WNV
c-yes siRNA 214 + WNV
c-yes siRNA 316 + WNV
Lamin A/C siRNA + WNV

Fig. 2B

K-means clusters of 238 genes with changes at any one time point post WNV(NY1999) infection

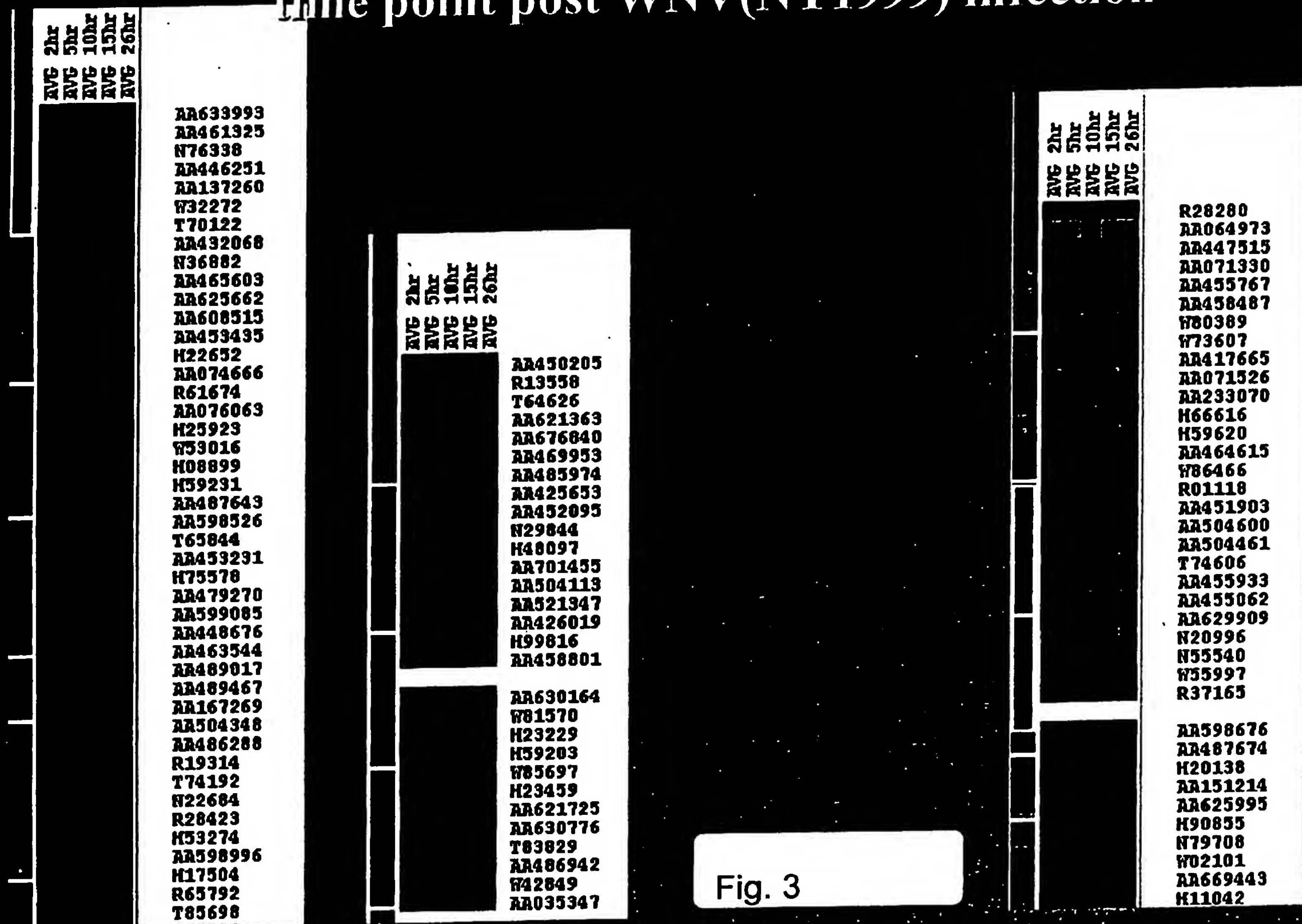
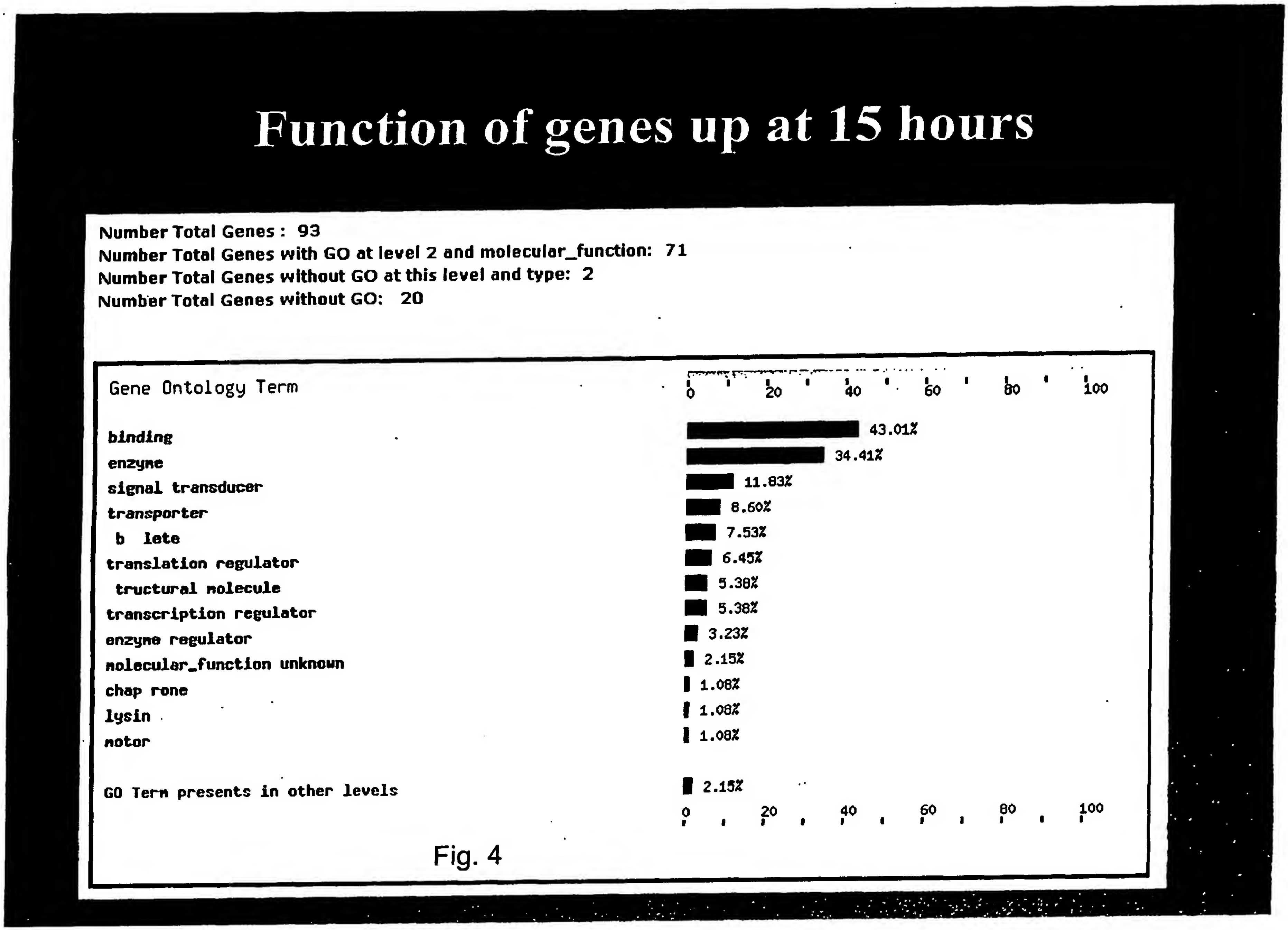


Fig. 3


Function of genes up at 15 hours
Number Total Genes : 93
Number Total Genes with GO at level 2 and molecular_function: 71
Number Total Genes without GO at this level and type: 2
Number Total Genes without GO: 20
Gene Ontology Term
0 20 40 60 80 100
binding 43.01%
enzyme 34.41%
signal transducer 11.83%
transporter 8.60%
b late 7.53%
translation regulator 6.45%
tructural molecule 5.38%
transcription regulator 5.38%
enzyme regulator 3.23%
molecular_function unknown 2.15%
chap rone 1.08%
lysin 1.08%
motor 1.08%
GO Term presents in other levels 2.15%
0 20 40 60 80 100
Fig. 4

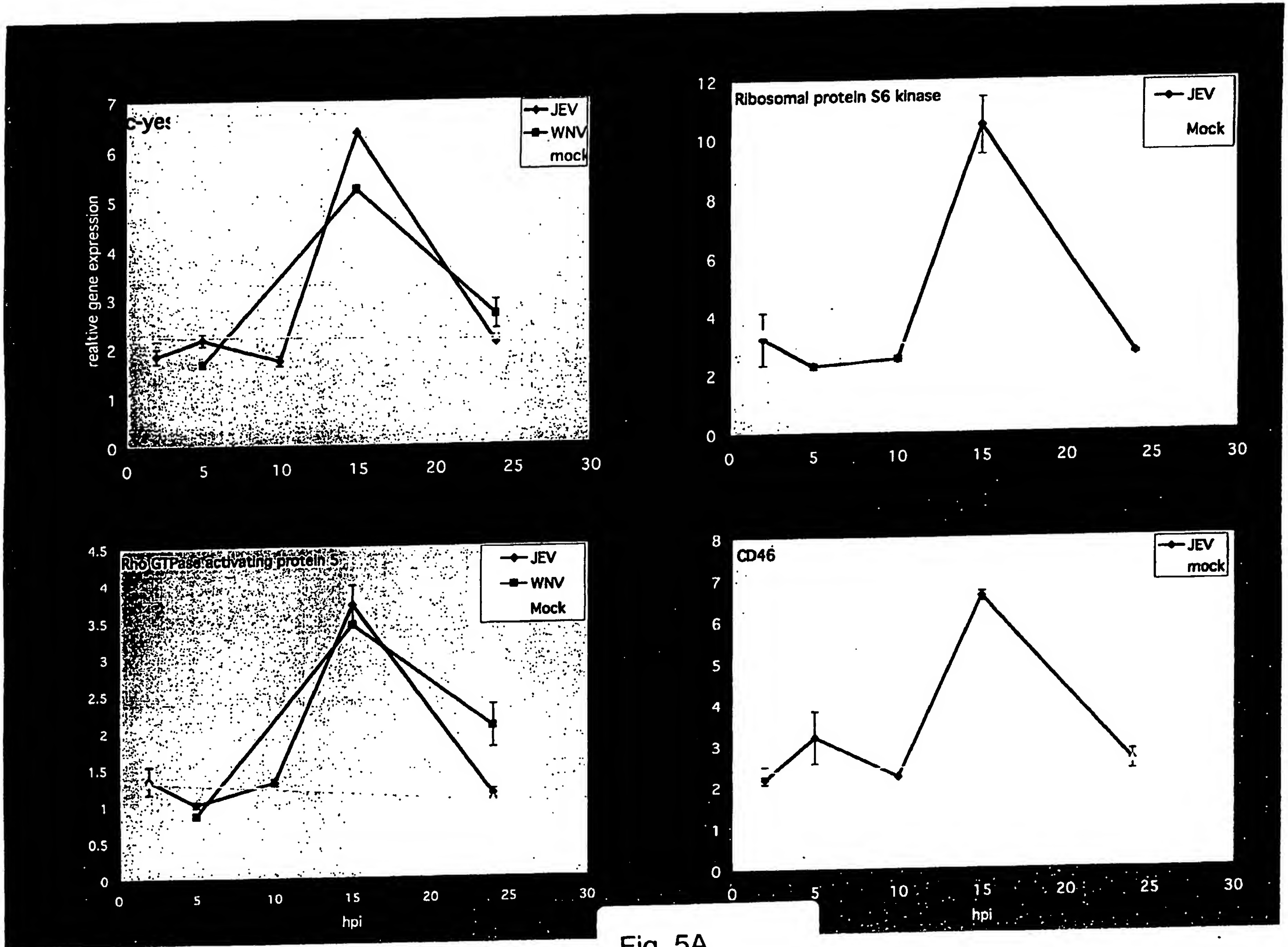

c-yes
JEV
WNV
mock
realtive gene expression
Ribosomal protein S6 kinase
JEV
Mock
Rho GTPase activating protein 5
JEV
WNV
Mock
hpi
CD46
JEV
mock
hpi

Fig. 5A

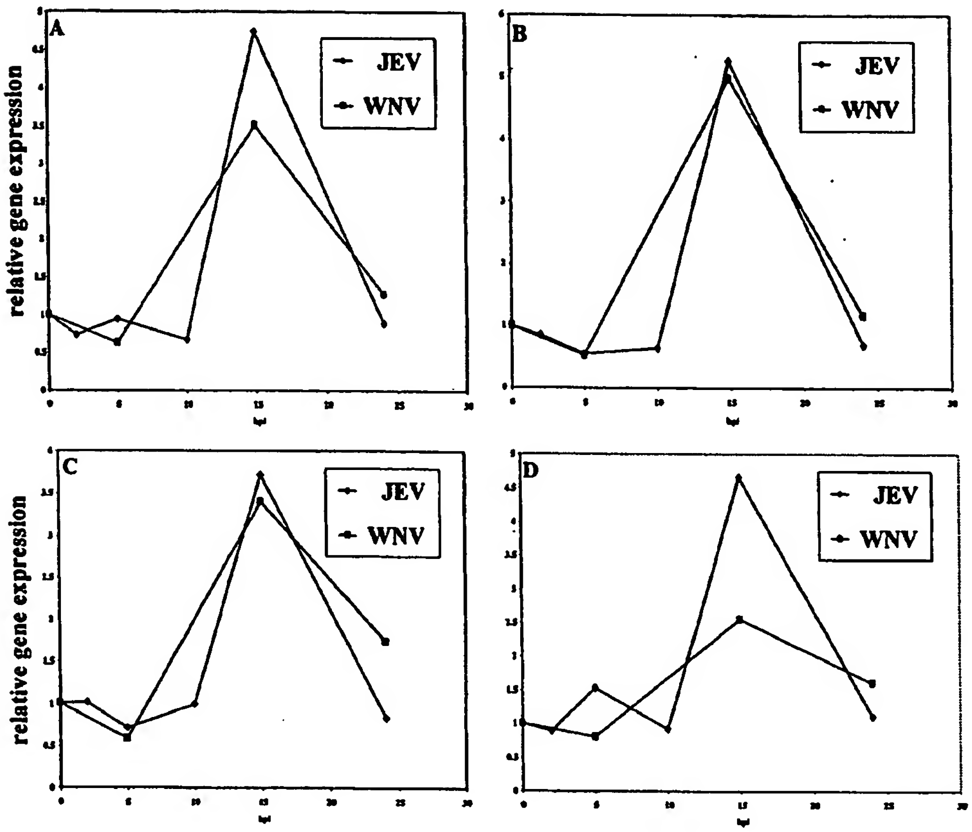

A
JEV
WNV
relative gene expression
B
JEV
WNV
C
JEV
WNV
relative gene expression
D
JEV
WNV

Fig. 5B

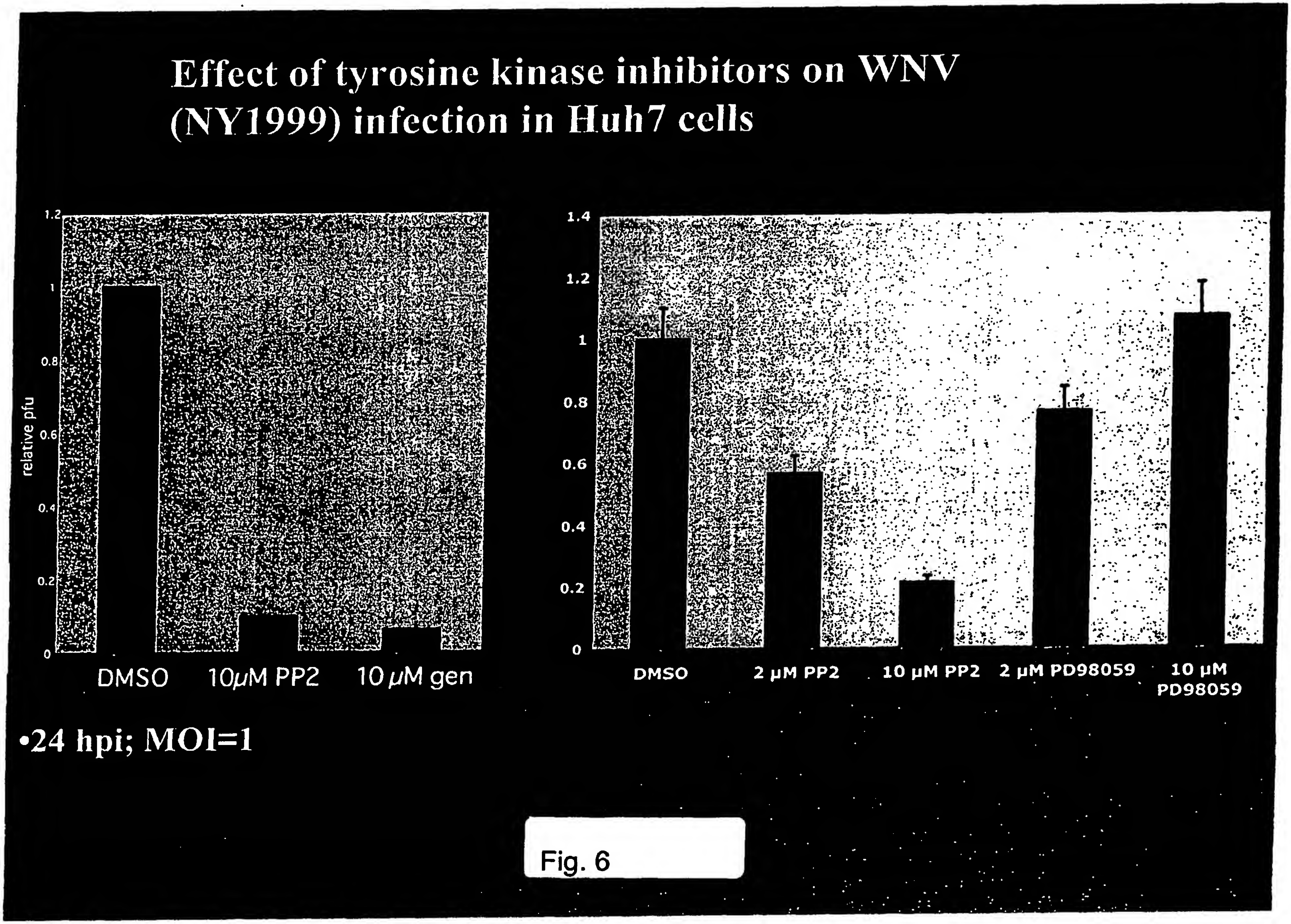

Effect of tyrosine kinase inhibitors on WNV (NY1999) infection in Huh7 cells
relative pfu
1.2
1
0.8
0.6
0.4
0.2
0
DMSO
10μM PP2
10 μM gen
1.4
1.2
1
0.8
0.6
0.4
0.2
0
DMSO
2 μM PP2
10 μM PP2
2 μM PD98059
10 μM PD98059
•24 hpi; MOI=1

Fig. 6

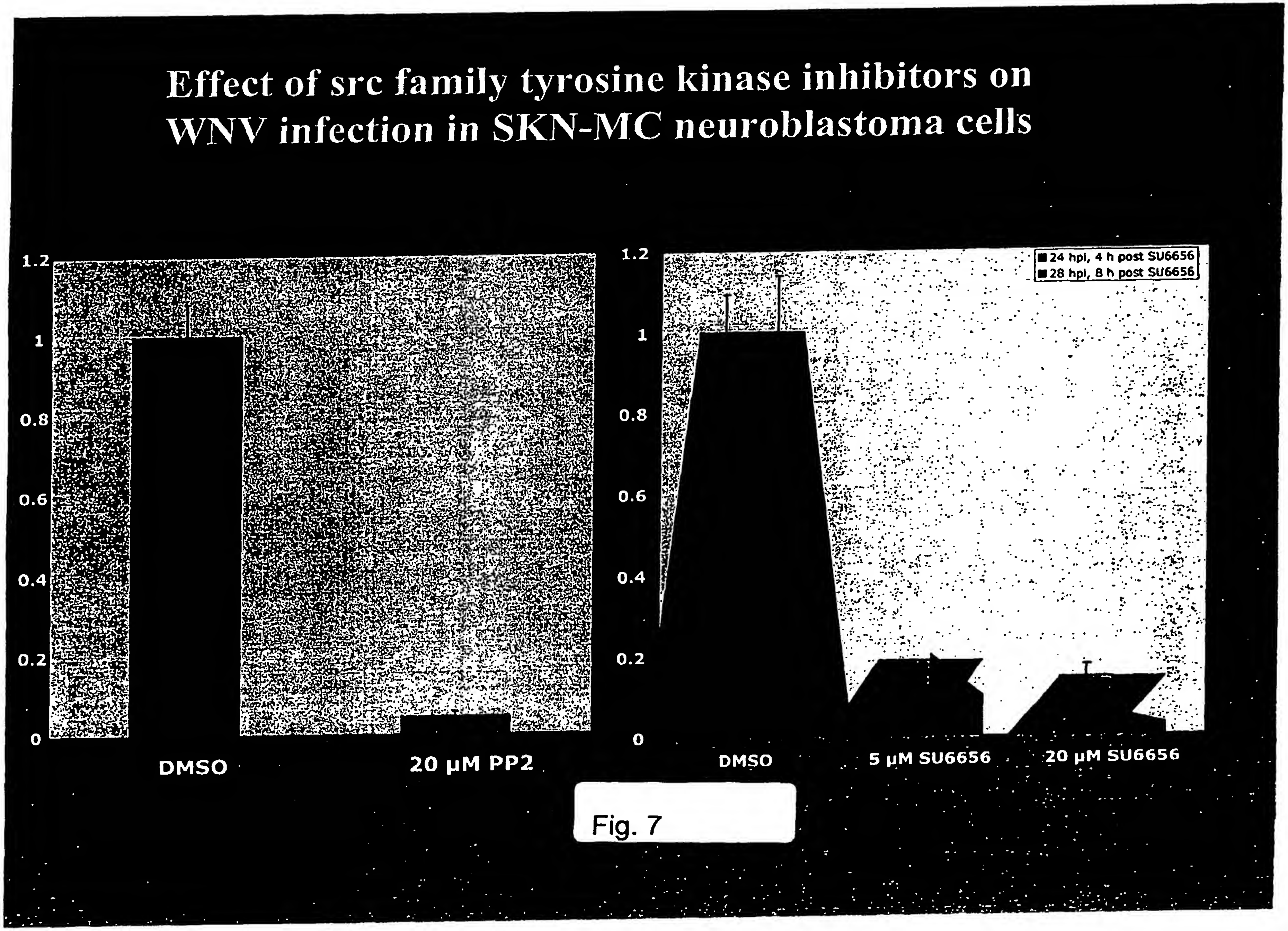

Effect of src family tyrosine kinase inhibitors on WNV infection in SKN-MC neuroblastoma cells
1.2
1
0.8
0.6
0.4
0.2
0
DMSO
20 μM PP2
24 hpl, 4 h post SU6656
28 hpl, 8 h post SU6656
DMSO
5 μM SU6656
20 μM SU6656

Fig. 7

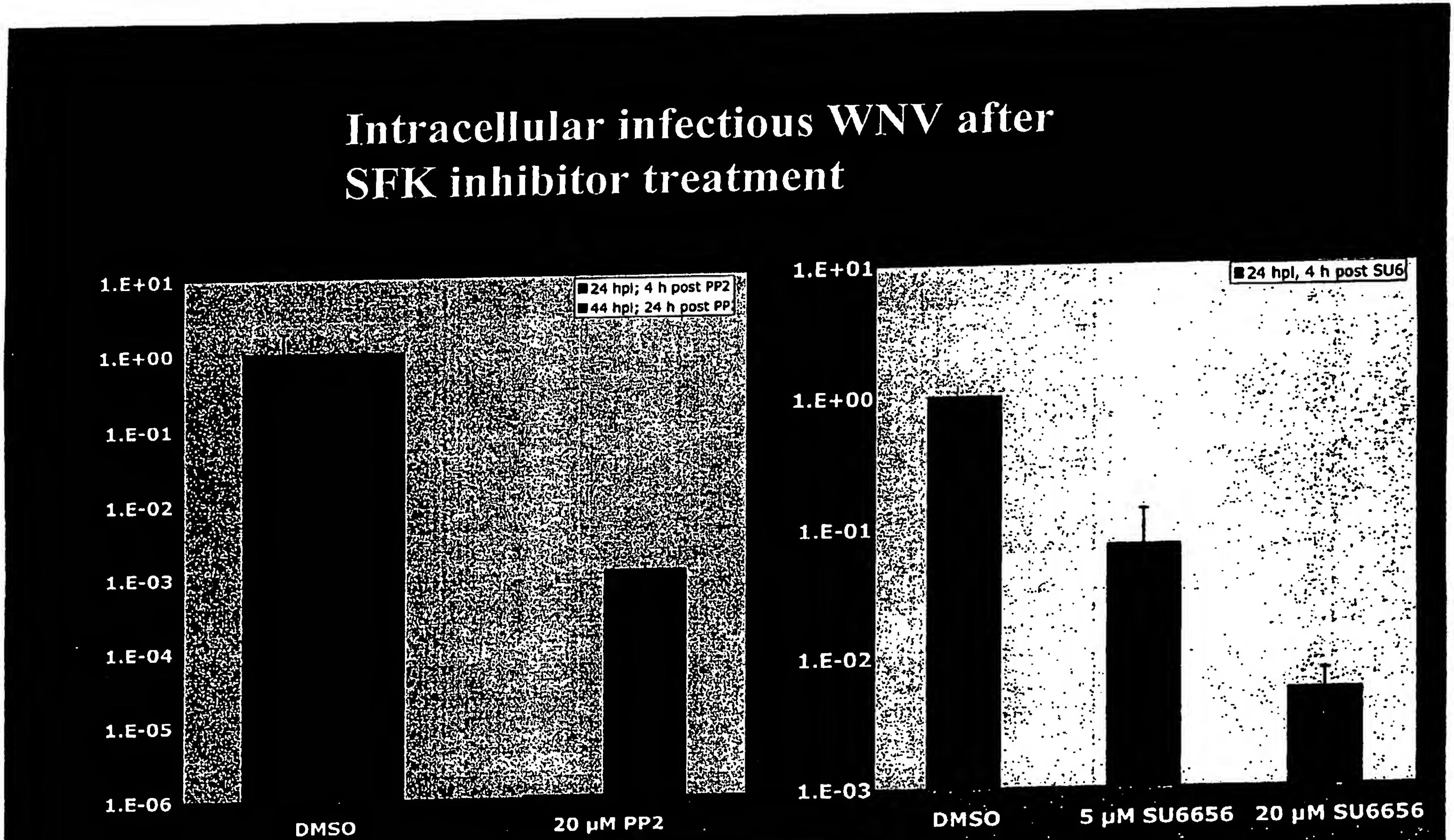

Intracellular infectious WNV after
SFK inhibitor treatment
1.E+01
1.E+00
1.E-01
1.E-02
1.E-03
1.E-04
1.E-05
1.E-06
24 hpi; 4 h post PP2
44 hpi; 24 h post PP
DMSO
20 μM PP2
1.E+01
1.E+00
1.E-01
1.E-02
1.E-03
24 hpi, 4 h post SU6
DMSO
5 μM SU6656
20 μM SU6656

Fig. 8

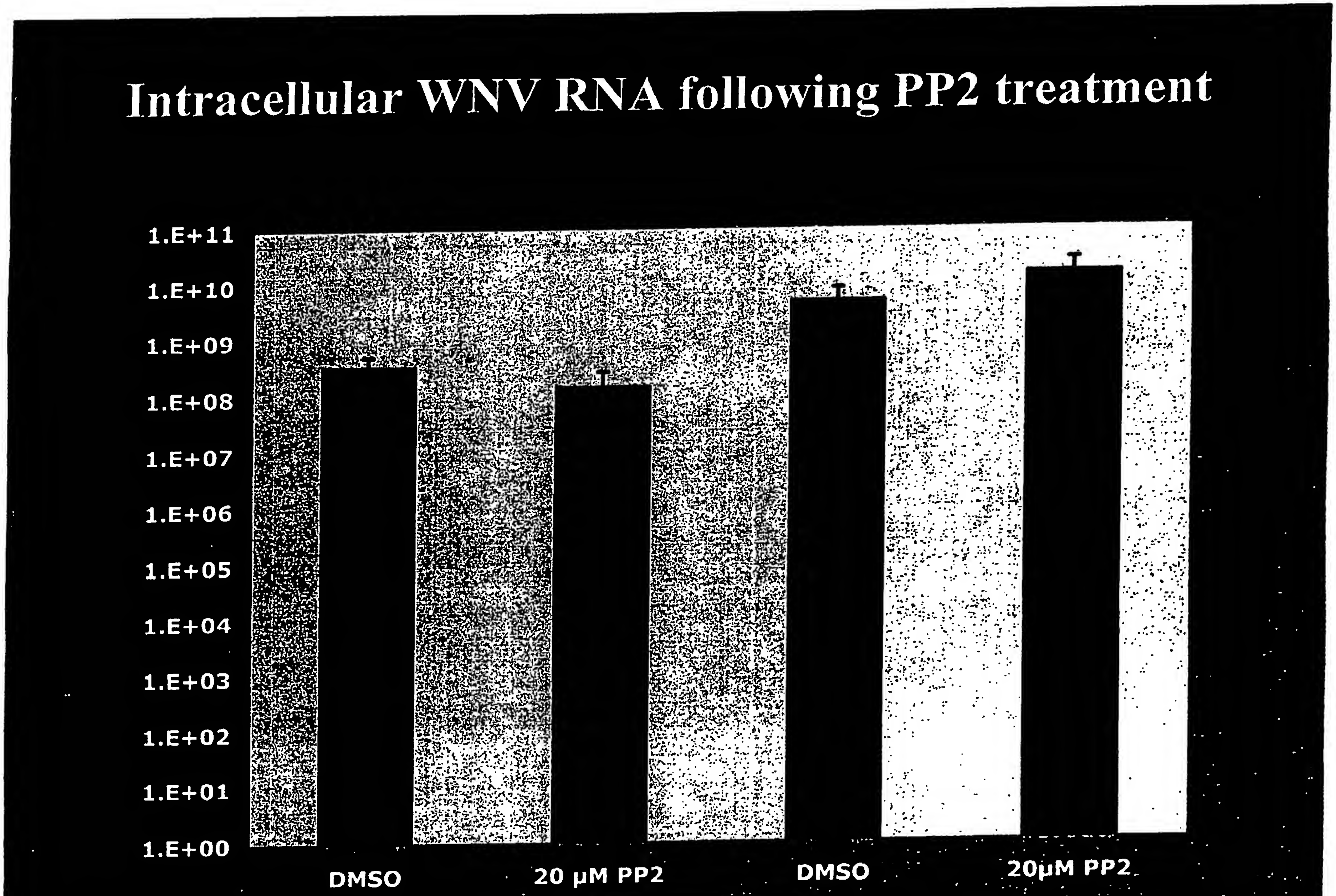
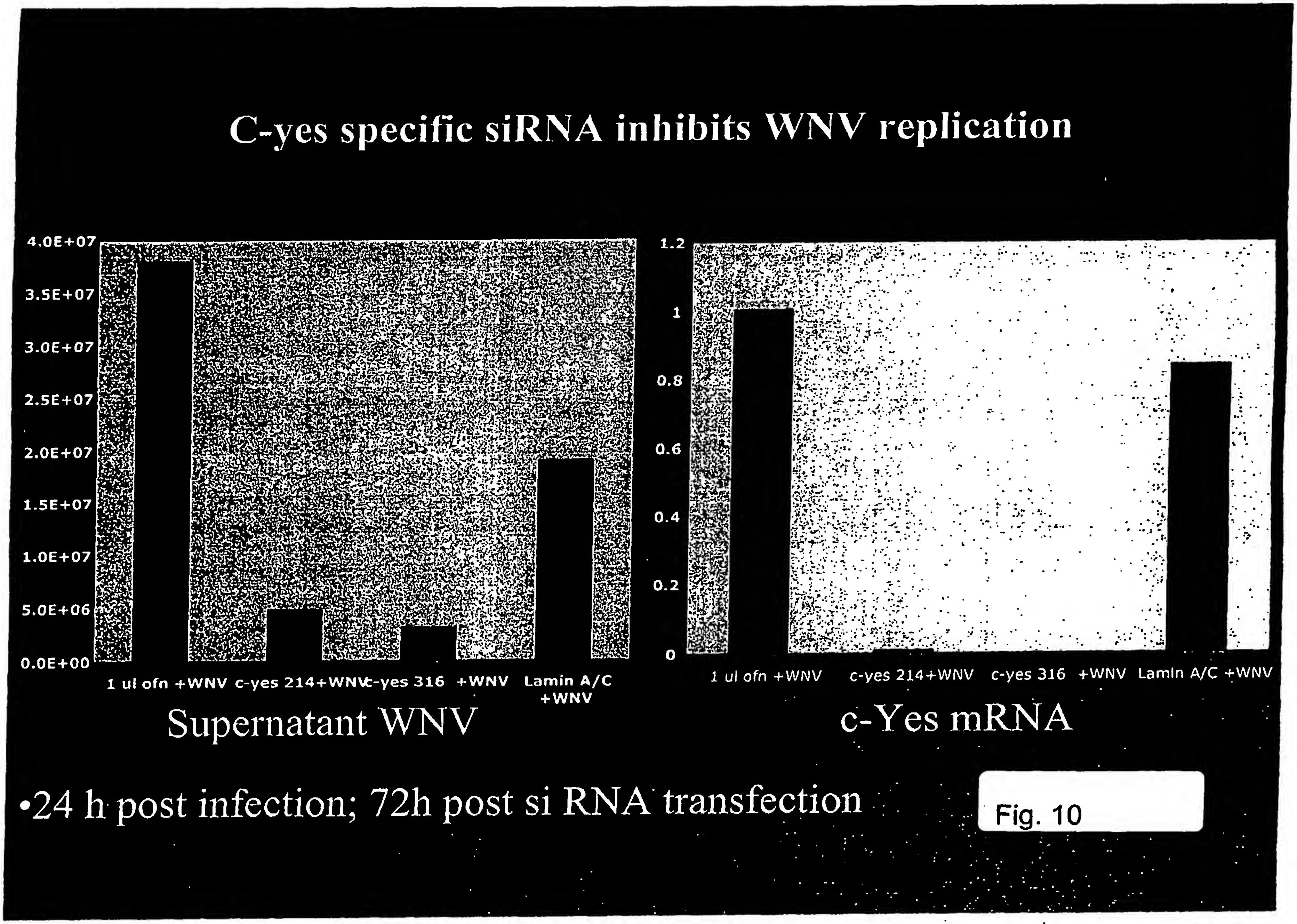

Intracellular WNV RNA following PP2 treatment
1.E+11
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
DMSO
20 μM PP2
DMSO
20μM PP2

Fig. 9


C-yes specific siRNA inhibits WNV replication
4.0E+07
3.5E+07
3.0E+07
2.5E+07
2.0E+07
1.5E+07
1.0E+07
5.0E+06
0.0E+00
1 ul ofn +WNV
c-yes 214+WNV
c-yes 316 +WNV
Lamin A/C +WNV
Supernatant WNV
1.2
1
0.8
0.6
0.4
0.2
0
1 ul ofn +WNV
c-yes 214+WNV
c-yes 316 +WNV
Lamin A/C +WNV
c-Yes mRNA
•24 h post infection; 72h post si RNA transfection
Fig. 10

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PP2

Size

Cat. No. 529573 — 1 mg

Synonym: 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine

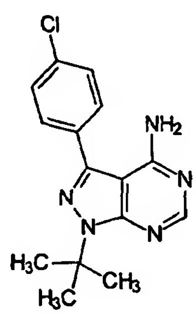
Description: A potent and selective inhibitor of the *src* family of tyrosine kinases similar to PP1. Selectively inhibits $p56^{lck}$ (IC_{50} = 4 nM), $p59^{fynT}$ (IC_{50} = 5 nM), and Hck (IC_{50} = 5 nM) compared to other tyrosine kinases, such as EGF-R (IC_{50} = 480 nM), JAK2 (IC_{50} >50 µM) or ZAP-70 (IC_{50} >100 µM). Also potently inhibits anti-CD3-stimulated tyrosine phosphorylation of human T cells (IC_{50} = 600 nM).

Form: Pale purple solid. Packaged under an inert gas.

Molecular Weight: 301.8

Molecular Formula: $C_{15}H_{16}ClN_5$

Structure:



Purity: ≥95% by HPLC

Solubility: DMSO. Further dilute with aqueous buffers just prior to use.

Storage: Freezer (-20°C). Following reconstitution, store in the refrigerator (+4°C). This product is stable for 3 years as supplied. Stock solutions are stable for several months at +4°C.

Reference: Hanke, J.H., et al. 1996. *J. Biol. Chem.* **271**, 695.

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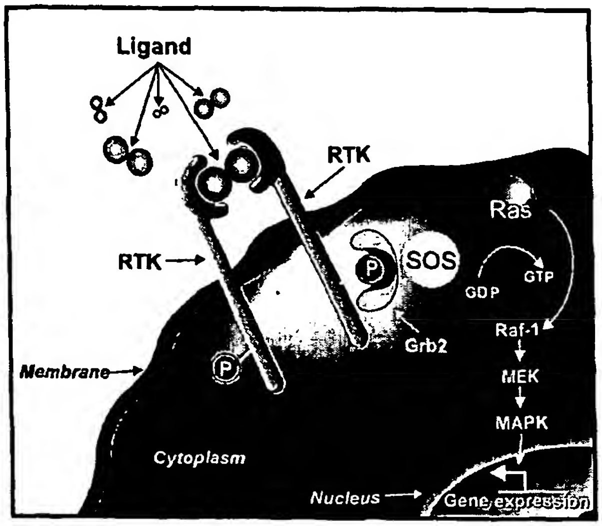
Protein Tyrosine Kinase Inhibitors

Protein tyrosine kinases mediate the transduction and processing of many extra- and intracellular signals. They are critical in regulating cell growth and differentiation and are deeply involved in oncogenesis. There are two general classes of protein tyrosine kinases: the receptor tyrosine kinases and the receptor-associated tyrosine kinases. The receptor tyrosine kinases possess an extracellular ligand binding domain and an intracellular catalytic domain with intrinsic tyrosine kinase activity.

Binding of a ligand to the receptor leads to a variety of downstream effects including stimulation of other tyrosine kinases, elevation of intracellular calcium levels, activation of serine/threonine kinases, phospholipase C and phosphatidylinositol-3´-kinase, and ultimately changes in gene expression.[1] Many substrates for protein tyrosine kinases contain a structural motif, Src homology 2 domain (SH2), that binds to phosphotyrosine residues and mediates the interaction of substrates with activated protein tyrosine kinases.[2,3] A model pathway leading to the activation of the MAP kinase cascade is depicted in the figure.



The ligand binds to the receptor tyrosine kinase (RTK) triggering receptor dimerization and autophosphorylation. Grb2 binds to the activated receptor via its SH2 domain and to Sos via its SH3 domain(s). Sos stimulates GDP-GTP exchange on Ras, activating a cascade of Ser/Thr kinases that ultimately leads to changes in gene expression.

The receptor-associated tyrosine kinases transmit signals from the membrane by interacting with the cytoplasmic domain of membrane proteins. One of the better characterized examples of this is the involvement of Lck in signaling through the T-cell receptor.[4]

The design of specific inhibitors of tyrosine kinases is important both for fundamental research and for developing therapeutic strategies for the treatment of disorders such as cancer, atherosclerosis, psoriasis, and septic shock in which increased tyrosine kinase activity has been reported.[5-7] Two classes of protein tyrosine kinase inhibitors have been developed. One acts by binding to the ATP binding site and the other by binding to the substrate binding site of the enzyme.

Among the inhibitors that act at the ATP binding site, genistein is the most commonly used. One drawback of this class of inhibitors is that they exhibit greater cytotoxicity and cause non-specific inhibition of serine/threonine kinases.[8]

Gazit and others have developed a series of synthetic compounds, tyrphostins, (also known as AG compounds), that inhibit protein tyrosine kinases by binding to the substrate binding site.[9-11] They structurally resemble tyrosine and erbstatin moieties and have hydrophobic characteristics which allow them to readily traverse the cell membrane. Many of the tyrphostins have selective and distinct inhibitory activities in various tyrosine kinase assay systems.

Cited References:
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PP2

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AG 1879
4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine

Off-white solid. PACKAGED UNDER INERT GAS. A potent and selective inhibitor of the Src family of protein tyrosine kinases. Inhibits $p56^{lck}$ (IC_{50} = 4 nM), $p59^{fynT}$ (IC_{50} = 5 nM), and Hck (IC_{50} = 5 nM). Does not significantly affect the activity of EGFR kinase (IC_{50} = 480 nM), JAK2 (IC_{50} > 50 μM), or ZAP-70 (IC_{50} >100 μM). Inhibits the activation of focal adhesion kinase and its phosphorylation at Tyr^{577}. Also potently inhibits anti-CD3-stimulated tyrosine phosphorylation of human T cells (IC_{50} = 600 nM). *Purity: ≥95% by HPLC.*

Ref.: Salazar, E.P., and Rozengurt, E. 1999. *J. Biol. Chem.* **274,** 28371; Hanke, J.H., et al. 1996. *J. Biol. Chem.* **271,** 695.

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Size	In Stock	Qty	Price
1 mg	Y		N/A

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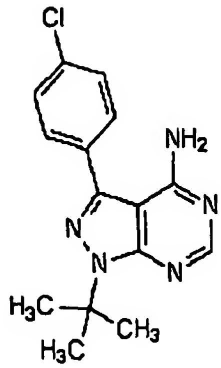
* NOTE: In Stock status is based on item availability worldwide.

Solubility	Molecular Formula	Mol. Wt.
DMSO	$C_{15}H_{16}ClN_5$	301.8



Additional Categories:

- Protein Kinases » Protein Kinase Inhibitors » Protein Tyrosine Kinase (PTK) Inhibitors

AG Compunds: Inhibitory Effects on Protein Tyrosine Kinases (IC_{50} and/or K_i in μM)

Product	Alternate Name	Cat. No.	EGFR-K poly CAT	ErbB-2/*neu* (HER1-2)-K	PGDFR-K	*trk*	InsR-K	$p60^{c-src}$-K	Comments	Ref.
AG 9	Tyrphostin A1	658390	>1250[a]						Negative control for other AG compounds.	1
AG 10	Tyrphostin A8	658475	560[a]						Inhibits GTPase activity of transducin (IC_{50} = 45 μM).	1,2
AG 17	Tyrphostin A9	658425	500[a]		0.5	>100			Selective inhibitor of PDGFR; uncouples oxidative phosphorylation.	1,3,4
AG 18	Tyrphostin A23	658395	40[a]		25	>100	4000	440	Potent broad range PTK inhibitor.	3,4
AG 30	Tyrphostin A30	121760							A potent protein tytosine kinase inhibitor that exhibits greater specificty for c-ErbB.	5,6
AG 43	Tyrphostin A63	658450	6500[a]						Negative control for other AG compounds.	1
AG 82	Tyrphostin A25	658400	3[a]			>100		150	Most commonly-used AG compounds.	1,2,3
AG 99	Tyrphostin A46	658430	10[a]				410		Selective for EGFR over InsR.	1,7
AG 112	Tyrphostin A48	658440	0.125[a]						Potent inhibitor of EGFR.	1
AG 126	Tyrphostin AG 126	658452	450[a]	>100	>100	>100			Inhibitor of LPS-induced tyrosine phosphorylation of $p42^{MAPK}$.	1,3,8
AG 183	Tyrphostin A51	658410	0.8[a]						Potent inhibitor of EGFR.	9
AG 213	Tyrphostin A47 (RG50864)	658405	2.4[a]		3	>100	640	8	Potent broad-range PTK inhibitor. Also inhibits PKC (IC_{50} = 60 μM).	9,10
AG 370	Tyrphostin AG 370	658454	>100[b]		25				Inhibits PDGF-induced mitogenesis in fibroblasts.	11
AG 490	Tyrphostin B42	658401	0.1[a]	13.5					Selective for EGFR over HER1-2. Also inhibits JAK-2.	7,9,12
AG 494	Tyrphostin B48	658407	1.24[a]	42	6		>100		Selective for EGFR over HER1-2.	7
AG 527	Tyrphostin B44(-)	658402	2.5[a]	37					Selective for EGFR over HER1-2.	7
AG 555	Tyrphostin B46	658404	0.7[a]	35			>100		Selective for EGFR over HER1-2.	7
AG 556	Tyrphostin B56	658415	5[a]	>500					Selective for EGFR over HER1-2.	7
AG 825	Tyrphostin AG 825	121765	19	0.35	40		>100		Highly selective for HER2-*neu* over EGFR.	13,14
AG 835	Tyrphostin B50	658409	0.86[a]						Positive enantiomer of AG 527.	7
AG 879	Tyrphostin AG 879	658460	>100[b]	1	>100	10			Selective inhibitor of $p140^{c-trk}$.	15,16
AG 957	Tyrphostin AG 957	121761	0.25						Selectively blocks the tyrosine kinase activity of human $p210^{bcr-abl}$ (K_i = 0.75 μM) over $p140^{bcr-abl}$ (K_i = 10 μM).	17
AG 1288	Tyrphostin AG1288	658510	>100	>100	>100				Blocks TNFα induced cytotoxicity.	8
AG 1295	Tyrphostin AG1295	658550	>100[b]		0.5				Highly specific inhibitor of PDGFR.	13,18
AG 1296	Tyrphostin AG1296	658551	>100[b,c]	>100	1.0			> 50	Highly specific inhibitor of PDGFR. Also inhibits FGF-induced proliferation (IC_{50} = 12 μM).	18
AG 1433	Tyrphostin AG1433	658553			5.0				Potent inhibitor of PDGF β-receptor kinase (IC_{50} = 5.0 μM) and KDR/Flk-1 (IC_{50} = 9.3 μM).	19,20
AG 1478	Tyrphostin AG1478	658552	0.003	>100	>100				Highly specific inhibitor of EGFR.	13
RG-1302		554725	1[b,c]						Shown to inhibit tumor growth in vivo.	21
RG-14620		554740	3[d]						Shown to inhibit tumor growth in vivo.	21
Bis-Tyrphostin		658418	0.4						Competitive inhibitor of the EGFR kinase.	4

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21. Yoneda, T., et al. 1991. *Cancer Res.* 51, 4430.

a= measurement of phosphorylation of an exogenous substrate
b= in vivo autophosphorylation assay
c= in vitro autophosphorylation assay
d= EGF-dependent cell proliferation assay

Abbreviations:
EGFR K = Epidermal growth factor receptor kinase
HER 1-2-K = Chimera of erbB-2/*neu* kinase with the ECF binding domain
InsR-K = Insulin receptor kinase
PDGFR-K = Platelet-derived growth factor receptor kinase
$p60^{c-src}$ = Cellular homolog of src gene protein
PTK = Protein tyrosine kinase

Other Selected Protein Tyrosine Kinase Inhibitors

Inhibitor	Cat. No.	M.W.	Comments	Ref.
Aminogenistein	155100	253.3	Inhibits tyrosine kinase $p56^{lck}$ phosphorylation of angiotensin I (IC_{50} = 1.2 μM).	1
Butein	203987	272.3	A plant polyphenol that inhibits EGFR tyrosine kinase (IC_{50} = 16 μM) and $p60^{c\text{-}src}$ (IC_{50} = 65 μM) activity.	2,3
Compound 32 (PD 153035)	234490	360.2	An extremely potent and specific inhibitor of the EGFR kinase (IC_{50} = 25 pM; K_i = 6 pM).	4,5
Compound 56	234505	388.3	The most potent and specific inhibitor of the EGFR kinase yet reported (IC_{50} = 6 pM).	4
Daidzein	251600	254.2	Inactive analog of genistein.	6
Damnacanthal	251650	282.3	Potent and reversible inhibitor of $p56^{lck}$ tyrosine kinase activity (IC_{50} = 17 nM for $p56^{lck}$ autophosphorylation and 620 nM for phosphorylation of an exogenous peptide). Does not significantly affect either PKA, PKC, or other tyrosine kinases.	7
Emodin	324694	270.2	Potent $p56^{lck}$ tyrosine kinase inhibitor (IC_{50} = 18.5 μM).	8
Erbstatin Analog	324930	194.2	Cell-permeable analog of erbstatin that inhibits the EGFR kinase (IC_{50} = 780 nM) and is stable in solution for >60 minutes.	9
Geldanamycin	345805	560.6	A potent inhibitor of $p60^{src}$ tyrosine kinase. Reported to destabilize mutated p53 protein from various cell lines.	10,11
Genistein	345834	270.2	Broad range tyrosine kinase inhibitor. Inhibits EGFR kinase (IC_{50} = 2.6 μM) and $p60^{v\text{-}src}$ (IC_{50} = 25 μM). Inhibits Ser/Thr kinases, PKC, and PKA (IC_{50} > 100 μM).	6
Herbimycin A	375670	574.7	Inhibits $p60^{c\text{-}src}$ function (IC_{50} = 900 μM) by irreversibly binding to the thiol groups of the kinase. Has known antitumor properties and has no significant effect on PKC or PKA activity.	12,13
HNMPA-$(AM)_3$	397100	454.4	Cell-permeable analog of HNMPA which yields the parent compound upon cleavage by cytosolic esterases. Inhibits insulin-stimulated glucose oxidation in intact cells (IC_{50} = 10 μM).	14
Lavendustin A	428150	381.4	Potent inhibitor of EGFR kinase (IC_{50} = 11 nM) and $p60^{c\text{-}src}$ (IC_{50} = 500 nM). Does not inhibit PKA or PKC at 100 μM.	15,16
Lavendustin B	428160	365.4	Inactive analog of lavendustin A. Inhibits the EGFR kinase (IC_{50} = 1.3 μM).	16
Lavendustin C	234450	275.3	Potent inhibitor of $p60^{c\text{-}src}$ (IC_{50} = 500 nM). However, it also inhibits Ca^{2+}/calmodulin kinase II (IC_{50} = 200 nM).	15
Lavendustin C Methyl Ester	234455	289.3	Inhibits EGFR kinase phosphorylation of RRSrc peptide (IC_{50} = 600 nM).	17
Leflunomide	429600	270.2	Immunosuppressive agent acts as an inhibitor of $p56^{lck}$, $p56^{lyn}$, JAK3, and EGFR kinase.	18
Piceatannol	527948	244.2	A plant metabolite. Preferentially inhibits the activity of Syk (IC_{50} = 10 μM), a non-receptor tyrosine kinase, over Lyn in isolated enzyme preparations.	19
PP2	529573	301.8	Potent and selective inhibitor of src family of tyrosine kinases. Inhibits $p56^{lck}$ (IC_{50} = 4 nM), $p59^{fynT}$ (IC_{50} = 5 nM), and Hck (IC_{50} = 5 nM). Does not affect the activity of EGFR kinase, JAK2, or ZAP-70 at these levels.	20
PP3	529574	211.2	A negative control for src family tyrosine kinase inhibitor PP2. However, it inhibits the activity of EGF receptor kinase (IC_{50} = 2.7 μM).	21
Radicicol, *Diheterospora chlamydosporia*	553400	364.8	Antifungal macrocyclic lactone that inhibits $p60^{c\text{-}src}$ kinase activity (IC_{50} = 8.2 μM). Inhibits the expression of mitogen-inducible COX-2 (IC_{50} = 27 nM) without affecting COX-1 expression in LPS-stimulated macrophages.	22,23
ST 638	567790	354.4	Inhibits phospholipase D activity in human neutrophils at a site between the receptor and the phospholipase.	24
SU1498	572888	390.5	A potent and selective inhibitor of Flk-1 kinase (VEGFR kinase) that exhibits only a weak inhibitor effect on PDGFR, EGFR, and HER-2 kinases.	25
SU4984	572625	333.4	Inhibits tyrosine kinase activity of the fibroblast growth factor receptor 1 (IC_{50} = 10 - 20 μM in the presence of 1 mM ATP). Reported to inhibit PDGF receptor and insulin receptor phosphorylation. Also inhibits aFGF-induced phosphorylation of ERK1 and ERK2 (IC_{50} = 20 - 40 μM). Does not inhibit the kinase activity of EGF receptor.	26
SU5402	572630	296.3	Inhibits the tyrosine kinase activity of the fibroblast growth factor receptor 1 (IC_{50} = 10 - 20 μM in the presence of 1 nM ATP). Acts as a weak inhibitor of PDGF receptor phosphorylation and does not inhibit the phosphorylation of insulin receptor. Also inhibits aFGF-induced phosphorylation of ERK1 and ERK2 (IC_{50} = 10 - 20 μM). Does not inhibit the kinase activity of EGF receptor.	26
SU5614	572632	272.7	A potent inhibitor of VEGF (Flk-1) (IC_{50} = 1.2 μM) and PDGF (IC_{50} = 2.9 μM) receptor tyrosine kinases. Does not affect EGF and IGF receptor tyrosine kinases.	27
Psi-tectorigenin	540100	300.3	Inhibits EGR receptor tyrosine kinase activity (IC_{50} = 3.3 μM). Also inhibits EGF-induced Ca^{2+} release and phosphatidylinositol turnover.	28,29
Tyrosine-Specific Protein Kinase Inhibitor	657015	2482.7	Peptide corresponding to the non-catalytic domain of $p60^{v\text{-}src}$ (137-157). Inhibits $p60^{v\text{-}src}$ (IC_{50} = 7.5 μM) and EGFR kinase but does not significantly inhibit PKA or PKC even at 100 μM.	30

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